The mechanism of platelet dysfunctions in stroke-prone spontaneously hypertensive rats (SHRSP) was investigated. Platelet aggregation was inversely correlated with blood pressure or heart weight/body weight ratios in various strains of spontaneously hypertensive rats (SHR), indicating genetic defects. Thrombin-induced 47 kDa protein phosphorylation was markedly reduced in platelets of SHRSP compared with that in Wistar-Kyoto (WKY) rat platelets, accompanying reduced aggregation and secretion, but in 20 kDa protein phosphorylation was unchanged. Ca\(^{2+}\) ionophore A23187-induced responses were also significantly decreased in SHRSP, and the degrees of the changes were greater than those by thrombin. However, 12-O-tetradecanoylphorbol 13-acetate-induced responses in SHRSP were similar to those in WKY rats, suggesting that protein kinase C activity and its substrate were normally present in SHRSP platelets. Phosphatidylinositol content in platelets of SHRSP was 20% less than that in WKY rat platelets, but the contents of other phospholipids, including phosphatidylinositol-4-monophosphate and phosphatidylinositol-4,5-bisphosphates, were unaltered. Thrombin-induced formation of diacylglycerols and phosphatidic acid did not differ from each other at the low concentrations. In the absence of Ca\(^{2+}\), thrombin-induced responses occurred to a similar degree in both platelets, whereas the enhancements by Ca\(^{2+}\) were much greater in WKY rats than in SHRSP. These results suggested that defective Ca\(^{2+}\) functions in receptor-mediated activation of protein kinase C and postkinase-mediated events appear to be an underlying mechanism for the hypofunctions in SHRSP platelets. (Hypertension 1989;14:304–315)
lets, but the causative defect would be in the impaired Ca\textsuperscript{2+} functions involved in the phosphorylation of P47 and in the reactions leading to physiological responses.

**Materials and Methods**

**Reagents**

\[ ^{14}\text{C}]\text{Serotonin (58.5 mCi/mmol) and} \]
\[ ^{3}\text{H}]\text{arachidonic acid (83.3 Ci/mmol) were obtained from} \]
\[ \text{New England Nuclear (Boston, Massachusetts);} \]
\[ ^{32}\text{P}]\text{orthophosphate was obtained from Japan Isotope Co. (Tokyo, Japan);} \]
\[ 12-\text{O-tetradecanoyl-phorbol 13-acetate (TPA) was from Sigma Chemical Company (St. Louis, Missouri);} \]
\[ \text{Ca}^{2+} \text{ionophore A23187 was from Calbiochem (San Diego, California);} \]
\[ \text{x-ray film was from Fuji film (Kanagawa, Japan);} \]
\[ \text{acrylamide monomer, N,N',N''-tetramethylethylene-bis-acrylamide, sodium dodecyl sulfate (SDS),} \]
\[ \text{N,N',N''-tetramethylenediamine, and Coomassie brilliant blue R250 were from Nakarai Chemicals (Kyoto, Japan);} \]
\[ \text{molecular weight (MW) protein standard for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was from Bio-Rad Laboratories (Richmond, California);} \]
\[ \text{standard phosphatidylinositol (soybean), PIP (bovine brain), PIP\_2 (bovine brain), and phosophatidic acid (PA) were from Sigma Chemical Company (St. Louis, Missouri);} \]
\[ 1,2-

**Experimental Rats and Measurement of Blood Pressure**

SHR, SHRS, and WKY rats were originally provided by Professor K. Okamoto of Kinki University Medical School and have been maintained by brother-sister breeding in our laboratory. Rats had free access to laboratory chow (MF, Oriental Yeast Co., Tokyo, Japan) and water. Male SHR and SHRS, aged 10–17 weeks unless otherwise described, and age-matched male WKY rats were used throughout this study. Mean blood pressure was measured by a tail-pulse pick-up method in unanesthetized rats after warming the body about 10 minutes in a chamber maintained at 37°C.

**Preparation of Washed Platelets and Measurement of Platelet Aggregation**

Washed platelets were prepared as previously described, principally according to the method of Feinstein et al.\textsuperscript{10} Platelet aggregation was measured in the presence of 1.5 mM Ca\textsuperscript{2+} at 37°C by a turbidimetric method that used a four-channeled NKK Hematracer 1 (Niko Bioscience, Tokyo, Japan) as described elsewhere.\textsuperscript{8}

\[ ^{14}\text{C}]\text{Serotonin Secretion} \]

The assay was performed by a modification of the methods of Hofmann et al\textsuperscript{11} and Holmsen and Dangelmeier\textsuperscript{12} as described previously.

**Analysis of Platelet Protein Phosphorylation**

The platelet protein phosphorylation experiment was performed principally by the method of Feinstein et al.\textsuperscript{10} Washed platelets, prepared according to the method of Feinstein et al\textsuperscript{10} and resuspended in buffer A (137 mM NaCl, 5.4 mM KCl, 0.2% glucose, 25 mM Tris-HCl, pH 7.4), were incubated with \[ ^{32}\text{P}]\text{orthophosphate (0.2 mCi carrier free to 2x10\textsuperscript{6} cells/ml) for 90 minutes at room temperature.} \]

**Analysis of Platelet Inositol Phospholipids**

To 1 ml washed platelet suspension (5x10\textsuperscript{9} cells/ml), 3.75 ml CHCl\textsubscript{3}:MeOH (1:2) was added. The mixture was left overnight at 4°C, shaken vigorously for 1 minute with 1.25 ml CHCl\textsubscript{3} and 1.25 ml 2 M KCl solution containing 5 mM EDTA, and centrifuged for 20 minutes at 3,000 rpm. The extracted phospholipids were resolved on a HPTLC plate as described previously.\textsuperscript{8} Phospholipids were eluted from silica with MeOH and analyzed for phosphorus according to the method of Bartlett.\textsuperscript{15}
FIGURE 1. Inverse correlation between platelet aggregation and blood pressure (Panel a) and between platelet aggregation and heart weight/body weight ratios (Panel b). Washed platelets from SHRSP (●), SHR (●), offspring of WKY rats and SHRSP (○), and WKY rats (□) at 18-20 weeks of age were stimulated with thrombin (0.22 units/ml) for 3 minutes at 37° C in the presence of 1.5 mM CaCl$_2$. WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats; SHRSP, stroke-prone spontaneously hypertensive rats.

CHCl$_3$:MeOH:2.5 M HCl (7:14:1.4) for PIP and PIP$_2$
and assayed for phosphorus by the method of Hess and Den.$^{19}$

Measurement of Diacylglycerol Formation

Platelet rich plasma (9 vol) was incubated with [H]arachidonic acid (8.33 μCi/ml in 10% BSA, 1 vol) for 60 minutes at 37° C.$^{20}$ Washed platelets labeled with [H]arachidonic acid (0.9 ml), which were resuspended in buffer B (25 mM Tris-HCl, 137 mM NaCl, 5.4 mM KCl, 0.2% dextrose, pH 7.4, 6×10$^8$ cells/ml), were incubated with thrombin at 37° C in the presence of 1.5 mM CaCl$_2$. Diacylglycerol was extracted and resolved by the method of Rittenhouse.$^{20}$

Measurement of Phosphatidic Acid Formation

Washed platelets (about 2×10$^9$ cells/ml) were incubated for 90 minutes at 37° C in equal volumes of neutralized [32P]orthophosphate (0.5 mCi/ml), and washed phosphorus-32-labeled platelets were resuspended in buffer B (about 4×10$^9$ cells/ml). Phosphorus-32-labeled platelets were stimulated with thrombin (20 μl) in the presence of 1.5 mM CaCl$_2$ (total volume 0.5 ml) at 37° C. PA formed was extracted and resolved by the method of Kennerly et al.$^{21}$

Assay of Protein

Platelet protein was measured by the method of Lowry et al.$^{22}$ BSA was used as the standard.

Results

Correlation Between Blood Pressure and Platelet Aggregation

Hypofunction of platelets in SHRSP was not secondary to circulation of degranulated platelets, which was observed in deoxycorticosterone acetate salt and renal hypertension,$^{23}$ nor could it be recovered by a long-term duration of hypertensive treatment.$^3$ To confirm whether the hypofunctions of platelets are due to genetic defects, the correlation of platelet aggregation and blood pressure was examined using SHR, SHRSP, WKY rats, and a cross from female SHRSP and male WKY rats at 18-20 weeks of age. These ages, a little older than other experiments, were chosen for this experiment because SHR develop high blood pressure more slowly than SHRSP. The blood pressure of the cross from SHRSP and WKY rats was similar to the range found in SHR. As Figure 1A shows, there was a strong inverse correlation between platelet aggregation and blood pressure ($r = -0.783$). An analogous inverse correlation ($r = -0.744$) was observed between platelet aggregation and heart weight/body weight ratio (Figure 1B). Together with other evidence,$^3,23$ these results indicate that genetic factors are involved in the observed dysfunctions of platelets in SHRSP.

Thrombin-Induced Protein Phosphorylation in Stroke-Prone Spontaneously Hypertensive Rat and Wistar-Kyoto Rat Platelets

Since protein phosphorylation in platelets precedes various physiological responses,$^6$ numerous reports have been presented on protein phosphorylation of human platelets. Before investigation on protein phosphorylation of platelets from SHRSP and WKY rats could be undertaken, we performed comparative studies of protein phosphorylation between human and rat platelets using the method applied on human platelets by Feinstein et al.$^{10}$ In
Figure 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of platelets labeled with $^{32}$P from stroke-prone spontaneously hypertensive rats (SHRSP) and Wistar-Kyoto (WKY) rats. Panel a: Coomassie blue staining from control platelets. Panel b and Panel c: autoradiographs. Platelets labeled with $^{32}$P from SHRSP and WKY rats at 16 weeks of age were stimulated for 30, 60, 120, and 180 seconds with thrombin (0.15 units/ml) in Panel b; in Panel c they were stimulated for 30, 60, and 180 seconds with Ca$^{2+}$ ionophore A23187 (0.5 μM). Molecular weights were determined using the following proteins as standard: lysozyme 14,400, soybean trypsin inhibitor 21,500, carbonic anhydrase 31,000, ovalbumin 45,500, bovine serum albumin 66,200, and phosphorylase B 92,500. W, Wistar-Kyoto rats; S, stroke-prone spontaneously hypertensive rats; 47K, 47 kDa protein; 20K, 20 kDa protein.

Platelets stimulated with thrombin (0.15 units/ml) for 1 minute at 37°C, P47 was strongly phosphorylated in both human and rat platelets. Twenty kilodalton protein (P20) phosphorylation was already observed in unstimulated rat platelets, and the change was only slight after stimulation whereas in human platelets phosphorylation of P20 occurred only on stimulation.

Figure 2 shows SDS-PAGE of phosphorus-32-labeled platelets from SHRSP and WKY rats.
when stimulated with thrombin (0.15 units/ml) for 30, 60, 120, and 180 seconds. Figure 3 illustrates the time courses and concentration-dependent curves of thrombin-stimulated [14C]serotonin secretion and aggregation and P47 and P20 protein phosphorylation. Aggregation responses were always measured with phosphorus-32-labeled platelets, prepared for protein phosphorylation, to confirm no artificial alteration during the labeling procedure. When phosphorus-32-labeled platelets (5×10^8 cells/ml) were stimulated with thrombin (0.15 units/ml) for various times at 37°C, P47 phosphorylation occurred instantly and reached a maximum at 30 seconds. The time course of phosphorylation was parallel to that of [14C]serotonin secretion. In Figure 3B, showing concentration-dependent curves, platelets were stimulated with thrombin (0.11, 0.22, 0.30, and 0.43 units/ml) for 3 minutes. Phosphorylation curve of P47 was consistent with aggregation and secretion curves. Phosphorylation of P20 was not concentration dependent.

**Ca²⁺ Ionophore A23187-Induced Protein Phosphorylation**

In human platelets stimulated with thrombin or other physiological stimuli, there was a rapid and transient rise in diacylglycerols and [Ca²⁺], after the hydrolysis of phosphatidylinositols. These two second messengers synergistically activate protein kinase C whose substrate is P47. The Ca²⁺ ionophore A23187 and several phorbol esters such as TPA mimic endogenous Ca²⁺ and diacylglycerol signals, respectively, bypassing receptors. To examine separately the two signals given by thrombin, the Ca²⁺ ionophore A23187 and several phorbol esters such as TPA were employed for the survey of calcium pathway. Effects of the Ca²⁺ ionophore A23187 on platelet functions and protein phosphorylation were comparatively shown in platelets of SHRSP and WKY rats (Figure 4). The Ca²⁺ ionophore A23187 (0.5 μM) induced [14C]serotonin secretion and aggregation in platelets of SHRSP to remarkably low degrees compared with those in

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**Figure 3.** Thrombin-induced [14C]serotonin secretion, aggregation, and protein phosphorylation in platelets from SHRSP and WKY rats. Panel a: time courses. Washed platelets (5×10^8 cells/ml) from SHRSP ( ) and WKY rats ( - - - - ), prelabeled with H as described in text, were stimulated for 30, 60, 120, and 180 seconds with 0.15 units/ml of thrombin in the aggregometer tubes at 37°C. [14C]serotonin prelabeled platelets (4×10^6 cells/ml) were stimulated with 0.5 units/ml thrombin. Results are expressed as mean±SD (n=4 in protein phosphorylation, n=3 in [14C]serotonin secretion). Panel b: concentration dependency curves. Platelets prelabeled with H or [14C]serotonin from SHRSP ( ) and WKY rats ( ) were stimulated with thrombin (0.11, 0.22, 0.30, and 0.43 units/ml) for 3 minutes. Results are expressed as mean±SD (n=4 in [14C]serotonin secretion, n=5 in aggregation) and mean of duplicates in protein phosphorylation. SHRSP, stroke-prone spontaneously hypertensive rats; WKY, Wistar-Kyoto rats; P47, 47 kDa protein; P20, 20 kDa protein.
WKY rat platelets. SDS-PAGE of phosphorus-32-labeled platelets from SHRSP and WKY rats, when stimulated with the Ca$^{2+}$ ionophore A23187 (0.5 µM), was shown in Figure 2C. P47 phosphorylation in platelets of SHRSP was about one third of that in WKY rat platelets. P47 phosphorylation reached a maximum at 30 seconds and then gradual dephosphorylation was observed. The degree of differences in secretion, aggregation, and P47 phosphorylation between platelets of SHRSP and WKY rats was much greater on Ca$^{2+}$ ionophore A23187 stimulation (Figure 4A) than on thrombin stimulation (Figure 3A). Figure 4B shows concentration dependency curves of the Ca$^{2+}$ ionophore A23187 when stimulated for 1 minute for protein phosphorylation, 3 minutes for $[^{14}C]$serotonin secretion, and 5 minutes for aggregation. Separated points in aggregation curves represent responses at 1 minute, which were scarcely observed at lower concentrations. $[^{14}C]$Serotonin release (at 3 minutes) induced by 0.10, 0.25, and 0.35 µM Ca$^{2+}$ ionophore A23187 was significantly higher in platelets of WKY rats than in those of SHRSP. Aggregation responses at 1 minute were scarcely observed with Ca$^{2+}$ ionophore A23187 (0.4 µM) in platelets of SHRSP. At 1 minute, WKY rat platelets aggregated in response to 0.4 µM Ca$^{2+}$ ionophore A23187. Aggregation induced by 5-minute stimulation with Ca$^{2+}$ ionophore A23187 (0.2, 0.3, and 0.4 µM) was also much greater in WKY rat platelets than in platelets of SHRSP. Ca$^{2+}$ ionophore A23187 did not induce protein phosphorylation at less than 0.2 µM, but at 0.4 µM, P47 and P20 protein phosphorylation was observed, and responses to it were greater in platelets of WKY rats than in those of SHRSP. The fact that the magnitude of differences in various responses to Ca$^{2+}$ ionophore A23187 between platelets of WKY rats and SHRSP was larger than in responses to thrombin may imply that calcium functions are impaired in platelets of SHRSP.

12-O-Tetradecanoylphorbol 13-Acetate-Induced Protein Phosphorylation

Although the magnitude of the difference between the two strains varies depending on the stimulants,
FIGURE 5. TPA-induced [14C]serotonin secretion, aggregation, and protein phosphorylation in platelets from SHRSP and WKY rats. Panel a: time courses. Platelets prelabeled with 32P or [14C]serotonin from SHRSP (—•—) and WKY rats (—O—) were stimulated with TPA (80 nM for aggregation and protein phosphorylation, 100 nM for [14C]serotonin secretion) for 30, 60, 180, and 300 seconds in the aggregometer tubes at 37 °C. Panel b: concentration dependency curves of platelets prelabeled with 32P or [14C]serotonin from SHRSP (—•—) and WKY rats (—O—) were stimulated with varying concentrations of TPA for 5 minutes ([14C]serotonin secretion and aggregation) and 1 minute (protein phosphorylation). Each point and vertical bar represent mean±SD (n=3). TPA, 12-O-tetradecanoylphorbol 13-acetate; SHRSP, stroke-prone spontaneously hypertensive rats; WKY, Wistar-Kyoto rats; P47, 47 kDa protein; P20, 20 kDa protein.

the significant reduction of thrombin- and Ca2+-ionophore A23187-induced P47 phosphorylation, as well as [14C]serotonin secretion and aggregation in platelets of SHRSP, suggests that protein kinase C per se, or receptor-mediated activation of protein kinase C in SHRSP, seems to be defective. As TPA directly activates Ca2+-activated, phospholipid-dependent protein kinase C, platelets from SHRSP and WKY rats were stimulated with TPA (Figure 5). TPA (80 nM) gradually induced [14C]serotonin secretion and aggregation without differences in the magnitude of responses between platelets of SHRSP and WKY rats (Figure 5A). Phosphorylation of P47 by TPA (80 nM) was peaked rapidly in 30 seconds. In contrast, P20 was phosphorylated slightly and slowly, which coincided with the study by Naka et al.25 Figure 5B shows platelet responses to varying concentrations of TPA. Five-minute stimulation with TPA-induced secretion and aggregation responses in a concentration-dependent manner; the curves are similar in platelets of WKY rats and SHRSP. One-minute stimulation with 10 nM TPA fully phosphorylated P47. Higher concentrations of TPA produced analogous results. TPA effect on P47 phosphorylation was seen at a concentration as low as 0.5 nM (data not shown). Again, platelets of SHRSP and WKY rats equally phosphorylated at P47. Phosphorylation of P20 was not evident. These results strongly indicate that protein kinase C activity and its substrate P47 were normally present in platelets of SHRSP and that the defective protein phosphorylation would be ascribed to incomplete receptor-mediated activation of protein kinase C.

Phospholipid Contents in Platelets From Stroke-prone Spontaneously Hypertensive Rats and Wistar-Kyoto Rats

Phospholipids extracted from platelets from SHRSP and WKY rats were resolved by TLC, and the phospholipid composition was compared between the platelets from the two strains. The total lipid phosphorus (mean±SD) was 8.67±1.71 (n=18) and 8.92±1.65 (n=17) µg phosphorus/mg platelet protein in SHRSP and WKY rats, respectively, which were not significantly different from each other. Although phosphatidylinositol is a minor phospholipid in the platelet membrane, the content in platelets of SHRSP was significantly lower by
FIGURE 6. Effects of TPA and Ca\textsuperscript{2+} ionophore A23187 on aggregation of platelets from SHRSP and WKY rats. Platelets from SHRSP (---) and WKY rats (-----) were stimulated with TPA or Ca\textsuperscript{2+} ionophore A23187 at the concentrations indicated in the figure. Each point represents mean of triplicate assays. TPA, 12-O-tetradecanoylphorbol 13-acetate; SHRSP, stroke-prone spontaneously hypertensive rats; WKY, Wistar-Kyoto rats.

Thrombin-Stimulated Diacylglycerol Formation

[3H]Arachidonic acid–labeled platelets were stimulated with thrombin to examine the formation of diacylglycerol. Diacylglycerol was extracted from platelets and resolved on TLC as described under Materials and Methods. Diacylglycerol formation in human platelets peaked at 15 seconds and then decreased. However, in rat (WKY) platelets, the formation of diacylglycerol peaked at 15 seconds as in human platelets, then decreased slightly at 30 seconds, gradually increasing afterward. The formation in response to various concentrations of thrombin was examined simultaneously with aggregation tracings. With lower concentrations of thrombin (less than 0.15 units/ml), no significant formation of diacylglycerol was observed in both platelets, although aggregation responses were different (SHRSP 31%, WKY rats 68%). In supramaximal doses of thrombin, which induced full aggregation in both platelets (SHRSP 80%, WKY rats 80%, no significance), diacylglycerol formation was apparently observed; it was lower in platelets of SHRSP than in WKY rat platelets. This diminished formation of diacylglycerol apparently reflects the lower content of phosphatidylinositol in platelets of SHRSP. Thus, it appears likely that reduced diacylglycerol formation from inositol phospholipids is not responsible for attenuated responses of thrombin-induced aggregation, secretion, and protein phosphorylation in platelets of SHRSP.

Thrombin-Induced Phosphatidic Acid Formation

To confirm that the initial step of platelet activation is not impaired, PA formation was examined in platelets of SHRSP and WKY rats. As diacylglycerol produced from inositol phospholipids is quickly transformed to PA, the formation of PA reportedly reflects in vivo phospholipase C activity.\textsuperscript{26} Platelets were labeled with \textsuperscript{32}P and rinsed. There was no difference in \textsuperscript{32}P uptake into platelets between the two strains. When phosphorus-32–prelabeled platelets were stimulated with thrombin (0.1 units/ml) for various times, PA formation increased linearly with similar rates in both platelets for 30 seconds, but afterward the rate became slightly lower in platelets of SHRSP than in those of WKY rats (16% less at 90 seconds in platelets of SHRSP compared with WKY rat platelets without significant difference). The concentration dependency of PA formation on stimulation (30 seconds) was examined simultaneously with aggregation tracings. There was no difference in \textsuperscript{32}P]PA formation between WKY rats and SHRSP at the examined concentrations of thrombin despite the significant difference in the aggregation response at the concentration of 0.13 units/ml thrombin (SHRSP 25%, WKY rats 86%). Thus, these results suggest that neither phospholipase C activity nor diacylglycerol formation are related to the defective protein phosphorylation in platelets of SHRSP.

Effect of 12-O-Tetradecanoylphorbol 13-Acetate and Ca\textsuperscript{2+} Ionophore A23187 on Aggregation

Figure 6 shows the synergistic effects of TPA and the Ca\textsuperscript{2+} ionophore A23187 on aggregation of platelets. As shown before, TPA (80 nM) induced aggregation similarly in platelets of both strains of rats,
but the response to Ca\(^{2+}\) ionophore A23187 was less in SHRSP than WKY rats (Figure 6, upper two figures). Subminimum doses of TPA (10 nM) or Ca\(^{2+}\) ionophore A23187 (0.05 \(\mu\)M) did not cause aggregation in both platelets (Figure 6, lower left). However, co-stimulation with TPA and Ca\(^{2+}\) ionophore A23187 in the subminimum concentrations induced aggregation; the responses were less in platelets of SHRSP than WKY rat platelets (Figure 6, lower right).

**Effect of 12-O-Tetradecanoylphorbol 13-Acetate and Ca\(^{2+}\) Ionophore A23187 on Protein Phosphorylation**

Platelets prelabeled with \(^{32}\)P from SHRSP and WKY rats were stimulated with suboptimum doses of TPA (10–20 nM) or Ca\(^{2+}\) ionophore A23187 (0.1–0.2 \(\mu\)M) for 1 minute. Platelet aggregation and protein phosphorylation in response to these stimulations are shown in Table 1. Independent stimulation with Ca\(^{2+}\) ionophore A23187 (0.1–0.2 \(\mu\)M) induced neither aggregation nor phosphorylation, whereas stimulation with TPA (10–20 nM) induced full phosphorylation of P47 equally in platelets from SHRSP and WKY rats without inducing aggregation. However, TPA (10–20 nM) and Ca\(^{2+}\) ionophore A23187 (0.1–0.2 \(\mu\)M) together induced both aggregation and phosphorylation of P47. Aggregation was less in SHRSP than in WKY rats, and the phosphorylation was similar in SHRSP and WKY rats. Thus, it is suggested that in platelets of SHRSP, Ca\(^{2+}\) does not function normally, first in activation of protein kinase C and second in the steps coupling protein phosphorylation to physiological responses.

**Ca\(^{2+}\) Dependency of Secretion, Aggregation, and Protein Phosphorylation in Stroke-prone Spontaneously Hypertensive Rat and Wistar-Kyoto Rat Platelets**

Effects of extracellular Ca\(^{2+}\) concentration on thrombin-induced \(^{14}\)C-serotonin secretion, aggregation, and protein phosphorylation were compared in platelets of SHRSP and WKY rats (Figure 7). Both platelets released \(^{14}\)C-serotonin to a similar degree in the absence of Ca\(^{2+}\). However, the enhancement of thrombin-induced secretion and aggregation by extracellular Ca\(^{2+}\) was remarkably greater in WKY rat platelets than in platelets of SHRSP. This difference in enhancement by extracellular Ca\(^{2+}\) was observed regardless of the type of stimulants, including Ca\(^{2+}\) ionophore A23187 and adenosine diphosphate (data are not shown). At prehypertensive ages (less than 4 weeks) there was no difference in the degree of Ca\(^{2+}\) enhancement between platelets from SHRSP and WKY rats. In contrast to secretion and aggregation, the magnitude of enhancement in malondialdehyde formation by Ca\(^{2+}\) in platelets of SHRSP was similar to that in WKY rat platelets at levels below 1 mM Ca\(^{2+}\), and the enhancement became greater in SHRSP than in WKY rats at levels over 1.5 mM Ca\(^{2+}\). Malondialdehyde formation is regarded as an indicator of phospholipase A\(_2\) and cyclo-oxygenase activities in platelets.27
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FIGURE 7. Effects of extracellular Ca\(^{2+}\) concentrations on thrombin-induced \[^{32}P\]serotonin secretion, aggregation, and protein phosphorylation in platelets from SHRSP and WKY rats. \[^{32}P\]serotonin prelabeled platelets (Panel a) and platelets prelabeled with \[^{32}P\] (Panel b and Panel c) from SHRSP (—•—) and WKY rats (—○—) were stimulated with thrombin (0.22 units/ml) for 3 minutes in Panel a, 0.13 units/ml for 1 minute in Panel b and Panel c in the presence of 2 mM EGTA (shown in separated points) and in varying concentrations of Ca\(^{2+}\) (0, 0.25, and 1.5 mM). Each point represents mean of triplicates. SHRSP, stroke-prone spontaneously hypertensive rats; WKY, Wistar-Kyoto rats.

Thrombin-induced P47 phosphorylation of platelets prelabeled with \[^{32}P\] from SHRSP was not noticeably different from that of WKY rat platelets in the absence of Ca\(^{2+}\) or in the presence of 1 mM EDTA (shown in separated points). As was seen in secretion and aggregation responses, the enhancement in protein phosphorylation by the presence of extracellular Ca\(^{2+}\) was less in platelets of SHRSP than in WKY rat platelets. This attenuated enhancement by Ca\(^{2+}\) in platelets of SHRSP was also demonstrated in Ca\(^{2+}\) ionophore A23187 stimulation. Thus, the results obtained here suggest Ca\(^{2+}\) functions are impaired that are concerned with secretion, aggregation, and protein phosphorylation, but not those in phospholipase A\(_2\), cyclo-oxygenase, or thromboxane A\(_2\) synthetase pathways.

Discussion

A marked reduction of aggregation as well as secretion was observed in platelets of SHRSP compared with those of age-matched WKY rats with the development of hypertension.\(^1\) The degree of abnormality was inversely correlated with the magnitude of hypertension in various strains of SHR. The observation of reduced responses to different proaggregators in platelets of SHRSP\(^1\) suggested that the defect appears to be distal to receptor occupation. The hypofunctions of platelets in SHRSP were revealed not only with washed platelets but also with platelets prepared by a gel-filtration procedure with platelet rich plasma and whole blood.\(^28\) These defects are completely different from those found in platelets from experimentally hypertensive rats\(^25\) and were not corrected by hypertensive treatment.\(^3\)

Platelets of SHRSP have a normal content of serotonin and adenine nucleotides at less than 20 weeks of age when the rats were killed for the present experiments. In spite of severe hypopaggregability and reduction of secretion in the presence of Ca\(^{2+}\), neither malondialdehyde nor thromboxane B\(_2\) formation were affected in platelets of SHRSP; they were rather enhanced in platelets of SHRSP with higher concentration of stimulants. Thrombin-stimulated rises in [Ca\(^{2+}\)]\(_i\) in platelets of SHRSP were slightly delayed, but the maximum [Ca\(^{2+}\)]\(_i\) was in the same range compared with that of WKY rats.\(^4\) These abnormalities observed in platelets of SHRSP resembled the defects in human platelets reported by Hardisty et al.\(^29\)

Accompanying reduced physiological responses, thrombin-induced phosphorylation of P47 was markedly decreased in platelets of SHRSP, whereas TPA-induced aggregation, secretion, and phosphorylation of the protein in SHRSP platelets were similar to those in WKY rat platelets. Thus, it is suggested that protein kinase C activity and its substrate P47 were normally present in SHRSP platelets, and the defective protein (P47) phosphorylation results from incomplete receptor-mediated activation of protein kinase C. Reduction of thrombin-induced P47 phosphorylation in platelets could possibly result from defects in receptor functions,\(^1\) phospholipase C activity,\(^2\) phospholipids of platelet membrane,\(^3\) protein kinase C activity,\(^4\) P47,\(^5\) and others.\(^6\) The possibility of receptor malfunction has been excluded in the case of platelets of SHRSP since hypofunctions were observed with any stimulants.\(^1\)

Phosphatidylinositol, PIP, and PIP\(_2\) are minor constituents of mammalian membranes. However, agonist-induced phosphoinositide breakdown functions as a signal-generating system.\(^6,30\) Phosphatidyl-inositol was 20% less in platelets of SHRSP while the contents of PIP and PIP\(_2\) were not signif-
significantly different between the two strains. In addition, phospholipase C activity is not apparently related to attenuated responses of aggregation, secretion, and protein phosphorylation in platelets of SHRSP. Ca\textsuperscript{2+} dependency experiments of aggregation, secretion, and protein phosphorylation (Figure 5) showed that hypofunctions of platelets of SHRSP were observed only in the presence of extracellular Ca\textsuperscript{2+}, or they were exaggerated in such conditions. Furthermore, Ca\textsuperscript{2+} ionophore A23187 stimulation demonstrates more clearly the hypofunctions of platelets of SHRSP than thrombin stimulation. Since the abnormalities were observed only in the presence of extracellular Ca\textsuperscript{2+}, the results obtained so far suggest that Ca\textsuperscript{2+} function is defective in platelets of SHRSP, first in the receptor-mediated activation of protein kinase C, and second in post-protein kinase C-mediated events coupling to physiological responses. Recently, it has been demonstrated that P47, a major substrate of protein kinase C, contains a potential Ca\textsuperscript{2+}-binding "EF hand" structure and a region that strongly resembles known protein kinase C phosphorylation sites.\textsuperscript{31}

Divergent results on abnormalities of platelet aggregation and secretion in spontaneously hypertensive rats have been reported. We have consistently observed hypofunctions of platelets from SHRSP\textsuperscript{1,5,23,28,32} and SHR.\textsuperscript{23} In addition, these hypofuncions were demonstrated with any of the platelet preparations\textsuperscript{28} (whole blood, platelet rich plasma, gel-filtered, and washed) and also with various aggregating agents.\textsuperscript{1} The results obtained by Hamet et al\textsuperscript{33} coincides well with ours. In contrast, Koutouzov et al\textsuperscript{34} and Huzoor-Akbar and Anwer\textsuperscript{35} have reported increased aggregation in platelets of SHR.

We first reported a preliminary result in 1985\textsuperscript{32} that P47 phosphorylation was reduced in platelets of SHRSP. In this paper the mechanism of the defective protein phosphorylation related to hypo-aggregability was investigated. In 1988, Huzoor-Akbar and Anwer\textsuperscript{35} reported an increase in thrombin-induced protein phosphorylation, and Koutouzov et al\textsuperscript{34} observed an increase in phospholipase C activity in thrombin-stimulated platelets of SHR. These results contradict ours that were obtained in platelets of SHRSP. These contradictory results may not be due to the difference between SHR and SHRSP because we have shown that hypofunctions of platelets are correlated with blood pressure in the strains of WKY rats, including SHR, offspring of WKY rats and SHRSP, and SHRSP. Our findings suggest that the defect is distal to receptor occupancy, whereas the observations by Huzoor-Akbar and Anwer\textsuperscript{35} and Koutouzov et al\textsuperscript{34} suggest defective thrombin receptors. We showed that WKY rats and another strain of Wistar rat (Wistar Mishima) show different responses to thrombin, probably due to the difference in the thrombin receptors.\textsuperscript{5} Therefore, these divergent findings on SHR might be ascribed to biological variability in WKY rats and SHR as discussed by Kurtz and Morris\textsuperscript{36} and Yamori.\textsuperscript{37}

In an attempt to find the etiology of hypertension, it is not important to note the apparent difference between spontaneously hypertensive rats and control rats, but it is important to demonstrate the genetic deviation in SHR related to high blood pressure. Although there are several interesting reports of in vitro experiments on protein kinase C in SHR platelets\textsuperscript{38} and phospholipase C in SHR aorta,\textsuperscript{39} our in vitro preliminary results on protein kinase C suggest that it is extremely difficult to draw a conclusion for in vivo phenomena from in vitro experiments, especially in protein kinase C.

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**KEY WORDS** • platelets • protein kinase C • protein phosphorylation • genetic hypertension • stroke-prone spontaneously hypertensive rats
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