Platelet Phospholipase C Activity in Salt-Dependent Hypertension

Isabelle Limon, Jocelyne Blanc, Sophie Koutouzov, Andreas Knorr, Philippe Meyer, and Pierre Marche

In spontaneously hypertensive rats (SHR), enhanced responsiveness of phospholipase C has been reported in various cells and tissues. In SHR and in some patients with essential hypertension particularly, the increased phospholipase C responsiveness of platelets has been described as involved in the hyperreactivity to thrombin. To determine the relation between such an enzymic abnormality and hypertension, the platelet phospholipase C activity was investigated in various models of experimental hypertension (i.e., in the Dahl salt-resistant and salt-sensitive strains inbred by John Rapp at Toledo, Ohio, SR/Jr and SS/Jr, respectively) fed either on a low or a high NaCl-containing diet, and in deoxycorticosterone acetate (DOCA)—salt hypertensive rats. In phosphorus-32—prelabeled platelets, phospholipase C was determined by measurement of the thrombin-induced [32P]phosphatidic acid formation; the labeling of the P47 protein with 32P was also measured. In parallel experiments, the platelet reactivity was assessed by measurement of the thrombin-induced serotonin release. Under thrombin (0.05–0.5 units/ml) stimulation, phospholipase C activity, [32P]P47 labeling, and serotonin release were significantly increased in SS/Jr rats fed a high NaCl diet compared with SS/Jr rats fed a low NaCl diet. NaCl-rich diet did not modify phospholipase C in SR/Jr rats. Platelet reactivity and phospholipase C responsiveness were also normal in DOCA-salt hypertensive rats compared with controls. These results suggest a lack of relation between phospholipase C activity and high blood pressure; they indicate moreover that the enhanced platelet reactivity and phospholipase C activity observed in SS/Jr rats fed on a high NaCl diet is not secondary to hypertension or to high NaCl intake and is likely of genetic origin, as already observed in SHR. In SS/Jr rats, however, the hyperactivity of the enzyme appears to be NaCl regulated and may be involved in the elevation of blood pressure. (Hypertension 1990;15:381–387)

Phospholipase C is an enzyme of major importance in the regulation of many cellular processes, cell contractility and cell growth in particular.1,2 Primary hypertension is associated with increased vascular tone and contractility and is related to an abnormal Ca2+ handling.3,4 Investigations of phospholipase C activity or of related biochemical events (e.g., phosphoinositide metabolism) in various models of hypertension have been therefore actively pursued (see References 5 and 6 for reviews). Evidence for increased phospholipase C activity has been obtained from studies in the vascular wall, in cultured aortic smooth muscle cells, and in blood platelets of spontaneously hypertensive rats (SHR).7–10 In platelets, an enhanced phospholipase C response to thrombin has been observed both in SHR and in some patients with essential hypertension.11–13 Such data indicated that in hypertensive subjects phospholipase C may indeed be involved in the platelet hyperresponsiveness to various agonists.11,14–16 We have attempted to study both the relation between the elevation of blood pressure and that of platelet phospholipase C reactivity, and also the manner whereby enhanced phospholipase C activity can be involved in the pathogenesis of hypertension. To this end, we have investigated phospholipase C in a hereditary and a mineralocorticoid model of sodium-dependent experimental hypertension: in Dahl salt-sensitive and salt-resistant (SS/Jr and SR/Jr, respectively) rats fed with a low or a high NaCl-containing diet, as well as in rats with hypertension induced by deoxycorticosterone acetate (DOCA)—salt. Platelets from these animals were examined both in their quiescent state and after stimulation with thrombin. Phospholipase C activity...
TABLE 1. Body Weight and Systolic Pressure of Dahl Salt-Resistant and Salt-Sensitive Rats Under Low or High NaCl Diet and of Deoxycorticosterone Acetate–Salt Hypertensive Rats and Controls

<table>
<thead>
<tr>
<th>Rat strain</th>
<th>Series</th>
<th>n</th>
<th>Weight (g)</th>
<th>BP (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR/Jr</td>
<td>Low NaCl diet</td>
<td>a)</td>
<td>6</td>
<td>286±7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b)</td>
<td>8</td>
<td>319±12</td>
</tr>
<tr>
<td></td>
<td>High NaCl diet</td>
<td>a)</td>
<td>7</td>
<td>263±12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b)</td>
<td>8</td>
<td>291±8</td>
</tr>
<tr>
<td>SS/Jr</td>
<td>Low NaCl diet</td>
<td>a)</td>
<td>7</td>
<td>350±4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b)</td>
<td>6</td>
<td>349±13</td>
</tr>
<tr>
<td></td>
<td>High NaCl diet</td>
<td>a)</td>
<td>8</td>
<td>290±9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b)</td>
<td>5</td>
<td>333±10</td>
</tr>
<tr>
<td>DOCA-NaCl</td>
<td></td>
<td></td>
<td>7</td>
<td>326±18</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td>9</td>
<td>424±14</td>
</tr>
</tbody>
</table>

Two distinct series of rats were prepared, as detailed in the Methods section. The rats of series noted a) were used for studying the thrombin-induced secretion of serotonin. The rats of series noted b) were used for studying 32P labeling of phospholipids and of proteins. BP, blood pressure; SR/Jr, Dahl salt-resistant rats; SS/Jr, Dahl salt-sensitive rats; DOCA-NaCl, deoxycorticosterone acetate–salt hypertensive rats.

was estimated by the measurement of thrombin-induced formation of phosphatidic acid (PA). Moreover, because the phosphorylation of a 47 kDa protein (P47), which is one of the substrates of protein kinase C in platelets, is involved in the triggering by thrombin of platelet physiological response such as serotonin secretion, the thrombin-induced phosphorylation of this protein was also determined. In separate experiments, the serotonin secretion was measured.

Methods

Animal Preparation

Male Dahl rats of the SS/Jr and SR/Jr substrains (inbred by John Rapp, Toledo, Ohio) were kindly provided by Bayer AG (Wuppertal, FRG). Two series of these rats were prepared. The first series, which was used for studying the thrombin-induced secretion of serotonin, was prepared as follows. At the age of 5 weeks, rats of each SS/Jr and SR/Jr strain were divided into two groups that, for 7–8 weeks, were given a low sodium diet (0.3% NaCl) or a high sodium diet (8% NaCl), respectively. All had free access to tap water. They were killed at 12–13 weeks of age. In the second series, which was used for phospholipid and protein investigations, the high NaCl diet contained 4% NaCl; both low and high NaCl diets were started when rats were 7 weeks old, and the animals were killed at the age of 12–14 weeks.

DOCA-salt hypertensive rats were prepared as follows. At 8–9 weeks of age, Wistar rats (CERJ, St Berthevin, France) were anesthetized with ether and nephrectomized unilaterally. Eleven days later, DOCA-salt treatment started; the rats were given free access to 1% saline for drinking. They were killed 6–7 weeks later. Control animals were unilaterally nephrectomized and had free access to tap water. Blood pressure and body weight of each animal were measured weekly; the final parameters are given in Table 1.

Preparation of Washed Platelets Labeled with Phosphorus-32 or [3H]Serotonin

The methods for blood withdrawal and radioactive labeling of platelets in platelet rich plasma and the washing procedures for isolation of phosphorus-32–labeled or [3H]serotonin-labeled platelets have already been detailed. The suspensions of washed platelets were adjusted to equal concentrations (3–5 × 10⁸ cells/ml) and kept at 37°C for an equilibration period of 45–60 minutes before starting the reactions. These were carried out as follows: 0.4 ml platelets, suspended in a Ca²⁺-free Tyrode's/HEPES buffer (NaCl, 137 mM; KCl, 2.6 mM; MgCl₂, 0.9 mM; glucose, 5.5 mM; HEPES, 5 mM; gelatin, 0.25%; pH 7.4) were placed into aggregometer cuvettes, stirred for 1 minute (1,000 rpm) at 37°C in the presence of CaCl₂ (1.3 mM final) and then exposed either to vehicle, for obtaining the values in unstimulated control platelets, or to thrombin for 20 seconds unless otherwise specified. For serotonin secretion measurements, imipramine (10⁻⁶ M final) was added together with CaCl₂ to prevent serotonin reuptake during the release reaction.

Assays

Serotonin secretion. Thrombin stimulation of [3H]serotonin-labeled platelets was terminated by transferring the suspension into tubes containing 100 μl ice-cold EGTA (0.1 M). Thrombin-induced platelet serotonin release was determined by assaying, after centrifugation, the appearance of [3H]serotonin in the platelet-free supernatant as described before. Thrombin-induced serotonin release was expressed as the percentage of maximum release (total releasable), which was obtained by stimulation...
by Laemmli. Gels were stained with Coomassie blue, destained, dried, and finally autoradiographed.

Resolution of phosphorus-32-labeled phosphatidylinositol 4,5-bisphosphate ([32P]PIP2). Because platelets incorporate [3H]inositol very poorly, thrombin-induced [32P]PA formation is accepted as one of the most sensitive indexes of platelet phospholipase C activity.

Results

Studies With SR/Jr and SS/Jr Rats

Despite a slight difference between the two experimental protocols used for rat preparation (see Methods section), the final characteristics of rats used for our experiments (Table 1) approximated those currently reported in the literature (see Reference 27 for review). Only minor differences in body weight or blood pressure were apparent between rats of a single group on different preparative regimes (Table 1) and such variations did not interfere with the results obtained (see below).

Unstimulated platelets. The incorporation of [3H]serotonin into platelets was similar between the four groups of rats as was the spontaneous serotonin release, which ranged between 1–2% of total serotonin content (results not shown). The low degree of spontaneous release is characteristic of resting platelets. We have previously established that our experimental conditions for prelabeling of platelets with [32P]orthophosphate yield phosphorus-32–labeled platelets whose lipids are at the isotopic equilibrium. The [3P] incorporation into lipids and the distribution of labeling between the various phosphoinositides and PA did not differ significantly of platelets with a very high dose of thrombin (5 units/ml) for 5 minutes.

Protein phosphorylation. Stimulation of phosphorus-32–labeled platelets with thrombin was terminated by transferring the cell suspension into polypropylene tubes containing 3.75 volume ice-cold chloroform/methanol/concentrated HCl (20/40/1, by volume). The phospholipids were extracted and analyzed as previously described. The inositol-containing phospholipids and PA were separated by thin-layer chromatography on silica gel–coated plates according to Jolles et al, localized by autoradiography, and scraped off the plates for scintillation counting. [32P]PA, which was almost undetectable in unstimulated platelets, increased rapidly after stimulation by thrombin as a consequence of phospholipase C-mediated hydrolysis of phosphorus-32–labeled phosphatidylinositol 4,5-bisphosphate ([32P]PIP2). Because platelets incorporate [3H]inositol very poorly, thrombin-induced [32P]PA formation is accepted as one of the most sensitive indexes of platelet phospholipase C activity.

Statistical Analysis

One rat was used per experiment. Each measurement was performed in duplicate. Variability was expressed as the mean±SEM and tests of significance were performed using the unpaired Student’s t test.
FIGURE 1. Line graphs showing dose-response studies of thrombin-induced serotonin secretion in platelets of Dahl salt-resistant (left panel) and salt-sensitive (right panel) rats fed on either a low salt or a high salt diet. [3H]Serotonin-labeled washed platelets were prepared as described in Methods and were stimulated with various doses of thrombin for 20 seconds. Results are expressed as percentage of maximum [3H]serotonin releasable obtained by stimulating platelets for 5 minutes with 5 units/ml thrombin (100%). Significant difference between values obtained with high salt vs. low salt diet: *p<0.05.

Among the four groups of rats (Table 2). [32P]PA values never exceeded 2% of total phosphorus-32-labeled lipids (Table 2) thus further indicating that cell suspensions comprise fully rested platelets. Total protein content and relative distribution (calculated from Coomassie-stained gels) were comparable among the four groups of rats and behaved similarly (results not shown). Likewise the incorporation of 32P into and its relative distribution between P20 and P47 (Table 2) did not differ significantly.

Thrombin-stimulated platelets. In [3H]serotonin-loaded platelets, the maximal serotonin secretion induced by thrombin (as defined in the Methods section) ranged between 70 and 80% of total [3H]serotonin content in platelets and did not differ significantly between the four groups of rats examined (results not shown). The study of thrombin-induced serotonin release is presented in Figure 1. In the rats of the SR/Jr substrain, the dose–response profile for platelets from rats fed a high NaCl diet was superimposable with that for platelets from rats fed the low NaCl diet (Figure 1, left panel). In contrast, in rats of the SS/Jr substrain the serotonin released by platelets from rats fed a high NaCl diet was significantly higher (at the thrombin doses of 0.2 and 0.3 units/ml) than that released by platelets of rats fed a low NaCl diet (Figure 1, right panel).

Preliminary experiments showed that the kinetics of thrombin-induced phosphorus-32-labeled lipid and protein changes in platelets from Dahl rats were similar to those from SHR and Wistar-Kyoto (WKY) rats. However, in all strains, the low degree and variability of alterations in [32P]PIP2 and [32P]phosphatidylinositol 4-phosphate (PIP) precludes such comparative studies. Therefore, as a more reliable index of platelet phospholipase C activity, we measured the thrombin-induced increase in [32P]PA.

Measurements of thrombin-induced [32P]PA formation (Figure 2) and 32P labeling of P47 (Figure 3) yielded the following data: 1) the dose–response pro-
files for platelets from both SR/Jr dietary groups were superimposable (Figures 2 and 3, left panels) and 2) the thrombin-induced \(^{32}\)P incorporated into P47 were greater in platelets from SS/Jr rats fed on a high NaCl diet than in those from rats fed on a low NaCl diet (Figures 2 and 3, right panel).

**Studies With Deoxycorticosterone Acetate–Salt Hypertensive Rats**

The body weight and blood pressure of hypertensive and normotensive rats are indicated in Table 1. Before stimulation, platelets from rats of both groups exhibited similar characteristics; they incorporated \(^{3}H\)serotonin to the same extent and did not differ in their capacity to incorporate \(^{32}\)P into the lipid classes presently investigated (results not shown).

After stimulation, no significant difference in the thrombin-induced \(^{3}H\)serotonin release between the DOCA hypertensive rats and their normotensive controls (rats that were solely unilaterally nephrectomized) could be detected (Figure 4). The effect of thrombin on \(^{32}\)P incorporation into platelets from these Wistar-derived hypertensive rats was also not significantly different from that in platelets from normotensive Wistar or Wistar-Kyoto rats (Figure 5). Figure 5 also presents the value obtained for platelets from SHR under similar experimental conditions.

In another series of experiments, the platelet reactivity to thrombin was studied in Wistar rats that were fed on 1% NaCl for 6 weeks (their blood pressure was 115 ±3 compared with 113 ±4 mm Hg for controls that drank tap water; \(n=5–8\), NS). The results (not shown) indicated that thrombin-induced \(^{3}H\)serotonin release in such salt-fed Wistar rats did not differ significantly from the control group.

**Discussion**

The various parameters investigated in quiescent platelets of SR/Jr and SS/Jr rats (irrespective of diet) and of DOCA-hypertensive rats compared with their controls, were found to be similar. Such observations, which have been already detailed for platelets from SHR and from essential hypertensive patients, with respect to their respective controls,²⁶,¹¹,¹³,²⁸ indicate that processes of serotonin uptake, phosphate incorporation, protein phosphorylation, and inositol lipid labeling as well as the pool size of phospholipids, adenosine triphosphate, and proteins were similar in the groups under comparison and were not influenced by either a high NaCl intake or a blood pressure elevation.

After stimulation by thrombin and regardless of parameter measured (serotonin secretion, \(^{32}\)P) incorporation, \(^{32}\)P) incorporation into phosphatidylinositol, \(^{32}\)P) labeling; left panels of Figures 1, 2, and 3, respectively), the following observations were made: 1) The dose–response profiles for platelets from SR/Jr rats were superimposable irrespective of the diet. 2) The dose–response profiles obtained with SS/Jr rats fed a high NaCl diet were shifted to the left, compared with those of rats fed a low NaCl diet (Figures 1, 2, and 3, right panels). All data values obtained for SS/Jr under low NaCl diet were in fact lower than the corresponding data for the three other groups of rats (which were themselves comparable) (Figures 1, 2, and 3).

Our results clearly demonstrate that the thrombin-induced serotonin secretion and phosphoinositide hydrolysis are enhanced in platelets from SS/Jr rats fed on a high NaCl diet compared with those from SS/Jr rats fed on a low NaCl diet. According to the mechanism whereby thrombin stimulates platelets,¹¹,¹⁷,²²–²⁵ these results strongly suggest that, in SS/Jr rats fed on a high NaCl diet compared with SS/Jr rats fed on a low NaCl diet, an increase in phospholipase C activity may account for the enhanced platelet reactivity to thrombin; however, the participation of an increased protein kinase C activity that would
account for the enhanced $^{32}$P labeling of P47, and hence for the augmented serotonin release, cannot be eliminated. The enhanced platelet phospholipase C activity suggested by the present findings in Dahl hypertensive rats extends the previous finding of an enhanced phospholipase C responsiveness in SHR and in some essential hypertensive patients.\textsuperscript{11-13} In the course of these studies, two series of rats (noted a and b in Table 1) have been used for serotonin secretion measurements and for $^{32}$P labeling of both PA and proteins, respectively. It is unlikely that the aforementioned findings could be accounted for by variations in blood pressure or body weight that existed between the two series (Table 1).

Platelet phospholipase C hyperresponsiveness to thrombin was not secondary to the blood pressure elevation, as indicated by the results obtained with DOCA hypertensive rats. Indeed, despite a considerable elevation of the blood pressure of these rats (Table 1), the platelet reactivity to thrombin as well as the phospholipase C activity, as expressed either by the serotonin secretion (Figure 4) or by $^{32}$P$\rceil$PA elevation (Figure 5), did not differ significantly from those of normotensive controls. The results obtained with DOCA rats indicate moreover that a high NaCl intake did not influence the platelet reactivity to thrombin. This conclusion was supported by data obtained in SR/Jr rats and in Wistar rats that received 1% NaCl in water.

The present investigation demonstrates that phospholipase C is insensitive to NaCl in both SR/Jr and DOCA-hypertensive Wistar rats. As the latter do become hypertensive, one may consider that phospholipase C is not directly involved in their development of high blood pressure. Phospholipase C activity was similar between SS/Jr and SR/Jr rats when both groups were fed on a high NaCl diet, which further indicates a lack of relation between phospholipase C activity and high blood pressure. Nevertheless enhanced phospholipase C responsiveness to thrombin was observed in SS/Jr rats fed a high NaCl diet as compared with SS/Jr rats fed a low NaCl diet, and this difference is paralleled by a marked difference in the blood pressures. In this respect, platelets from SS/Jr rats fed on a high NaCl diet behave as those from SHR. It is noteworthy that increased platelet phospholipase C activity might be observed in those rat strains that are genetically prone to develop hypertension.

Phospholipase C is a key enzyme involved in cellular Ca$^{2+}$ translocation and cell growth or reactivity. In platelets of hypertensive subjects, the hypersensitivity of phospholipase C, through the enhanced production of the second messenger diacylglycerol and hence the hyperactivation of protein kinase C, appears responsible for the increased Na$^{+}$-H$^{+}$ exchange rate that has been claimed to be involved in the pathophysiology of primary hypertension.\textsuperscript{29-32} Moreover, in SHR phospholipase C hyperresponsiveness has also been reported in the vasculature, and as such is likely to participate in the increased proliferative response of smooth muscle cells.\textsuperscript{7-10} and hence in the vascular hypertrophy associated with hypertension.\textsuperscript{33} In addition, the hyperreactivity of platelets could also, through the release of vasoactive substances or growth factors (e.g., serotonin, platelet-derived growth factor) and their action on the vascular wall, participate in the enhanced vascular tone of hypertensive subjects. In the rat strains genetically hypertensive or predisposed to become hypertensive, it is conceivable that an increased phospholipase C activity might be a primary event involved in the increase in total peripheral resistance and in blood pressure elevation.

References
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