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RELEASE OF IMMUNOREACTIVE-NEUROPEPTIDE Y BY RAT PLATELETS

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Summary: Neuropeptide Y, a potent vasoconstrictor and cardiac depressant, is re-leased from sympathetic nerve endings. Its presence in megakaryocytes suggests this peptide might be stored in platelet granules and released during aggregation. Immunoreactive-neuropeptide Y was measured in platelet rich and platelet poor plasma, and was substantially greater in the former. Addition of collagen to platelets resulted in release of neuropeptide Y which paralleled, in a concentration-dependent manner, the degree of platelet aggregation. Adenosine diphosphate, at concentra-tions which induce only the first phase of aggregation and not the release reaction, caused only a minor release of neuropeptide Y. These results suggest that platelet release could be a major source of circulating neuropeptide Y and could contribute to hemodynamics of pathophysiological states involving platelet activation.

Neuropeptide Y is a 36 amino acid peptide with a widespread distribution in the mammalian nervous system, co-localized in norepinephrine-containing granules (1,2). It is also found in the heart (3,4) and the chromaffin cells of the adrenal medulla (5,6). Its levels rise in the blood during periods of active sympathetic stimulation (7,8) or intense stress or exercise (7,9). In the cardiovascular system, neuropeptide Y is a potent vasoconstrictor and also has negative inotropic and chronotropic effects on the myocardium (10).

Recent immunohistochemical studies of rat bone marrow have demonstrated de novo synthesis of this peptide in megakaryocytes (11,12). It has been hypothesized that megakaryocyte-derived neuropeptide Y is stored in platelets and released during platelet aggregation, and that platelet-derived neuropeptide Y might contribute to vasoconstriction and myocardial depression during intravascular thrombosis. However, the release of this substance during platelet aggregation has not been previously demonstrated. In the present study, we examined the release of neuropeptide Y by platelets following stimulation by adenosine diphosphate and collagen. We further investigated the possible involvement of neuropeptide Y in the aggregatory process.

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MATERIALS AND METHODS

Animals: Sexually mature male albino rats were used in this experiment. Rats were anesthetized with sodium pentobarbital (60 mg/kg, ip), and blood was collected from the abdominal aorta into ice-chilled tubes containing 3.8% sodium citrate (one part anticoagulant to nine parts blood), and aprotinin (1.0 trypsin inhibitory unit/ml blood). Platelet rich plasma and platelet poor plasma were prepared as previously described (13). Platelet count in PRP was determined by phase contrast microscopy.

Platelet Aggregation Studies: Aggregation of platelets was initiated by adding various doses of collagen (Hormon Chemie, Munich, FRG) or adenosine diphosphate (Sigma, St. Louis, MO) and was measured by the turbidimetric method of Born (14). The aggregation curves were recorded for 2 min with ADP and 5 min with collagen, periods corresponding with observed maximum aggregatory responses. At this time point approximating the maximum response at high concentrations of the stimuli, platelet rich plasma samples were removed from the aggregometer, rapidly centrifuged at high speed, and the supernatant aliquoted and stored at -70°C for neuropeptide Y determination. The aggregation tracings were analyzed to determine percent aggregation (using platelet poor plasma as the 100% standard and platelet rich plasma as the 0% standard) and the maximum slope of the response (in arbitrary units of cm/min), two common measures of platelet aggregation.

Additional aggregation experiments were performed in which platelets were preincubated with 10^{-8} to 10^{-4} M neuropeptide Y (Penninsula Lab., Belmont, CA) before initiation of aggregation by adenosine diphosphate or collagen, in order to measure any inhibitory or stimulatory action of the peptide on agonist-induced platelet aggregation. The direct effect of neuropeptide Y on platelets was also measured by adding high concentrations of the peptide to platelet rich plasma.

Plasma Neuropeptide Y-immunoreactivity: Neuropeptide Y was measured after cold acid-ethanol extraction of plasma samples, by radioimmunoassay as described previously (15), with $[^{125}I]$-labeled neuropeptide Y (Amersham) as the tracer. The neuropeptide Y antiserum used (Penninsula Lab.) has neglible cross-reactivity to peptides of similar size and structure. In addition to measurement in the plasma samples taken during platelet aggregation, the peptide was quantified in platelet rich plasma which was not aggregated, but in which platelets were disrupted during the extraction procedures. In further determinations, neuropeptide Y-immunoreactivity was measured in platelet poor plasma, in plasma prepared directly from whole blood by standard methodology (13), and in buffer containing resuspended platelets collected from the pellet after high speed centrifugation of platelet rich plasma.

Statistics: Results were expressed as mean and standard error. The platelet aggregation responses, measured either as percentage of maximal aggregation (with platelet rich plasma as the 0% standard and platelet poor plasma as the 100% standard), or as maximum slope of the aggregation curve (cm/min), and neuropeptide Y levels were compared between various experimental groups by the Bonferonni method for multiple comparisons, with statistical significance assumed at $p<0.05$.

RESULTS AND DISCUSSION

Immunoreactive-neuropeptide Y was present at significantly higher levels in platelet rich plasma than in either plasma prepared by standard methodology, or platelet poor plasma prepared by high speed centrifugation of platelet rich plasma ($p<0.01$, Table 1), suggesting the presence of the peptide in platelets. In control experiments (data not shown), addition of platelet rich plasma serial dilutions to the assay resulted in a parallel shift of the standard curve. The levels observed in plasma collected by standard methodology and in platelet poor plasma are comparable to those previously measured in rat plasma (15,16), whereas the levels in platelet rich plasma were in excess of one order of magnitude higher. Platelet pellets prepared from platelet rich plasma and resuspended in buffer also contained high levels of immunoreactive-neuropeptide Y (Table 1). The presence of high concentrations of neuropeptide

Table 1. Neuropeptide Y-immunoreactivity in standard rat plasma, platelet poor plasma prepared from platelet rich plasma, and platelet rich plasma. Each value represents the mean (± standard error) of 7 to 9 observations.

	Neuropeptide Y (pmol/ml)
Plasma[1]	0.41 ± 0.09
Platelet poor plasma	0.51 ± 0.13
Platelet rich plasma[2]	5.72 ± 0.37**
Resuspended platelet pellet[2,3]	7.85 ± 0.77**

**p<0.01, compared to plasma or platelet poor plasma.
[1]prepared by standard methodology directly from whole blood.
[2]neuropeptide Y levels are adjusted for a platelet count of 10^6/ul.
[3]pellet from recentrifuged platelet rich plasma, resuspended in equal volume buffer.

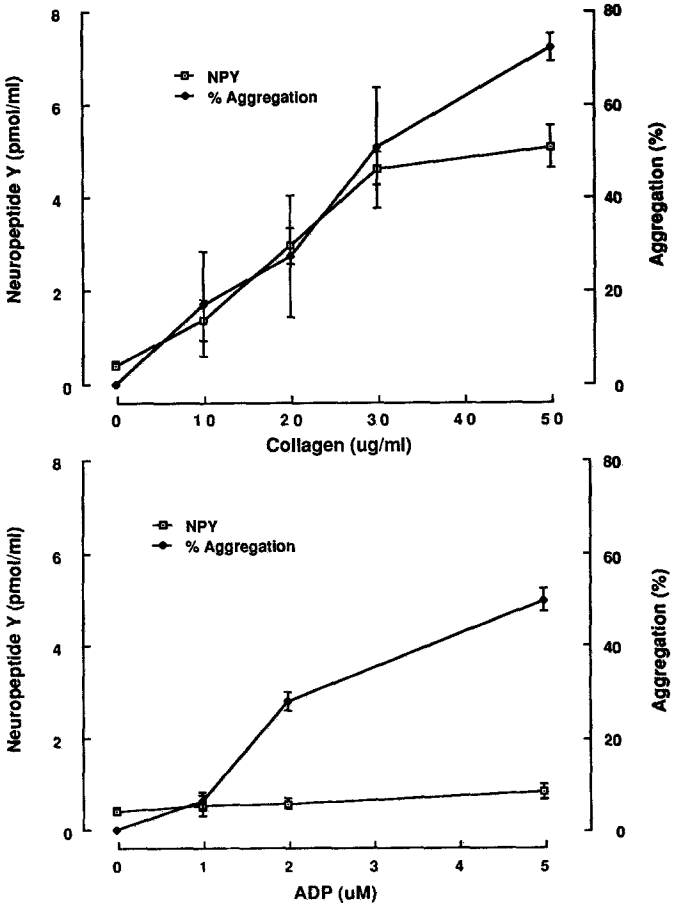

Figure 1. Aggregation and immunoreactive-neuropeptide Y release induced in rat platelet rich plasma by collagen (top, n=7) and ADP (bottom, n=9). Horizontal bars indicate standard errors.

Y in platelets and in platelet rich plasma suggests that this substance might be released from platelets during aggregation, and we therefore undertook studies to measure levels in plasma after platelet aggregation.

Stimulation of rat platelets with collagen resulted in a remarkably parallel aggregation response and release of immunoreactive-neuropeptide Y, as measured in platelet poor plasma prepared rapidly from the aggregating platelet suspension (Figure 1, top). When adenosine diphosphate was used as the platelet stimulus, only a minor release of immunoreactive-neuropeptide Y occurred (Fig. 1, bottom). These results demonstrate that the release of neuropeptide Y-immunoreactivity takes place during the platelet release reaction, because collagen stimulates irreversible platelet aggregation and the release reaction, whereas adenosine diphosphate, at the concentration tested, produced only primary, reversible aggregation. Primary aggregation induced by adenosine diphosphate is not associated with the platelet release reaction in citrated rat platelets (16,17).

Incubation of the rat platelet rich plasma with neuropeptide Y alone for periods of up to 30 min had no aggregatory effect, at concentrations of 10^{-8} to 10^{-4} M (data not presented). This lack of direct stimulatory effects of neuropeptide Y was also observed when human platelet rich plasma was tested in a few experiments.

When rat platelets were incubated with neuropeptide Y (10^{-8} to 10^{-4} M) or its vehicle before addition of 15 ug/ml collagen, or 2 or 5 uM adenosine diphosphate, no statistically significant effect of the peptide on aggregation was observed, either in terms of potentiation or inhibition (Table 2). Similar results were obtained when 25 ug/ml collagen was used to stimulate aggregation (data not shown). A tendency toward potentiation by neuropeptide Y of

Table 2. Effects of neuropeptide Y on rat platelet aggregation induced by collagen and adenosine diphosphate. Each value represents the mean (± standard error) of 4 or 5 observations.

Neuropeptide Y (M)	Aggregating Agent	Aggregation (%)	Slope of Aggregation (cm/min)
0	Collagen, 15 ug/ml	61±3.4	9.0±1.5
10^{-8}	"	59±5.5	9.8±3.0
10^{-7}	"	66±2.6	9.3±1.3
10^{-6}	"	61±2.5	8.5±1.3
10^{-5}	"	65±3.0	11.0±1.5
0	Adenosine diphosphate, 2 uM	10±1.9	3.3±0.5
10^{-7}	"	12±2.8	3.7±0.8
10^{-6}	"	17±4.2	4.4±0.6
10^{-5}	"	10±1.7	3.8±0.4
10^{-4}	"	12±3.2	3.7±0.6
0	Adenosine diphosphate, 5 uM	38±4.6	14.5±1.8
10^{-7}	"	42±5.2	17.0±1.3
10^{-6}	"	37±6.9	13.8±2.3
10^{-5}	"	33±6.2	13.3±2.3
10^{-4}	"	34±7.8	13.3±2.8

aggregation induced by the low concentration of adenosine diphosphate was observed, but this effect was not statistically significant (Table 2).

These results demonstrate the release of immunoreactive-neuropeptide Y from platelets during the second phase of aggregation. The release of neuropeptide Y from sympathetic nerve endings during their stimulation in isolated organs is well-documented (8), but under some circumstances in which the entire sympathetic nervous system is activated, a lack of correlation between catecholamine and neuropeptide Y levels has been observed (9,15,18). Platelet release of neuropeptide Y could represent a major source of the circulating peptide in pathophysiological states associated with platelet activation, including thrombosis, many forms of shock, and myocardial infarction. Because neuropeptide Y is a cardiodepressant and a vasoconstrictor in various vascular beds including the coronary, the possibility that platelet-derived neuropeptide Y might contribute to hemodynamic and cardiac events occuring during ischemic and thrombotic states should be investigated further.

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