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ISOLATION AND CHARACTERIZATION OF PEPTIDES WHICH ACT ON RAT PLATELETS, FROM A PHEOCHROMOCYTOMA

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Summary: An increase in the cellular concentration of cAMP leads to the inhibition of platelet aggregation. We have been investigating endogenous peptides which inhibit platelet function, using an assay which detects increase in platelet cAMP. Compared with the human adrenal medulla, a pheochromocytoma (PC) contained abundant peptides that elevate platelet cAMP. About 90% of the activity was found in the SP-III fraction which contained strongly basic peptides. From the SP-III fraction, peptides P-1, P-2 and P-3 were purified to homogeneity as endogenous peptides which elevated platelet cAMP. A gas phase sequencer was used to identify these peptides as follows: P-1=vasoactive intestinal peptide (VIP); P-2=calcitonin gene related peptide-I (CGRP-I); P-3=CGRP-II. It is well known these peptides are potent vasorelaxants. VIP and CGRP-I significantly increased platelet cAMP levels 15- and 6-fold, respectively. These results suggest that VIP and CGRP-I and -II act upon platelets as well as upon vascular tissue.

Aggregation of blood platelets is believed to be a critically important event both in normal blood hemostasis and in the development of thrombosis. The increase in the cellular cAMP level, achieved by several extrahumoral factors including prostacyclin and prostaglandin E_1 (PGE_1), leads to the inhibition of platelet aggregation (1). Thus, in order to prevent thrombus formation, prostacyclin and PGE_1 have been clinically used in cardio-bypass surgery (2) and in certain peripheral vascular disorders (3).

It has been reported that platelets have several types of receptors for peptides (4). To clarify the intricate mechanism of platelet function, it is important to identify peptides that act upon platelets. We have been

ABBREVIATIONS: PC, Pheochromocytoma; TFA, trifluoroacetic acid; RIA, radioimmunoassay; HPLC, High performance liquid chromatography; PTH, phenylthiohydantoin; VIP, vasoactive intestinal peptide; CGRP, calcitonin gene related peptide.

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investigating endogenous peptides which inhibit platelet function using an assay which detects increases in platelet cAMP. Pheochromocytoma (PC) is a tumor which stems from chromaffin cells and elicits several clinical features including hypertension and palpitation (5). It is well known that PC produces not only chatecholamines but several peptides. Adrenorphine (6) and neuropeptide Y (7) have been isolated from PC tissue.

We found that a peptide produced from PC elicited increased cAMP in rat platelets. Furthermore, we systemically investigated the endogenous peptides that elevated platelet cAMP.

Materials and Methods

Materials: Bovine Serum Albumin (Sigma, fraction V) was used after two recrystallizations. cAMP and succinyl (suc) cAMP Tyrosine methyl ester were purchased from Sigma. Synthetic peptides were purchased from Peninsula Laboratory, Inc. (California).

Radioimmunoassay for cAMP: cAMP antibody was prepared in our laboratory acording to the described methods (8), and ^{125}I-Succinyl-cyclic AMP Tyrosine methyl ester was prepared by the peroxidase method and purified by RP-HPLC as described (9). The incubation buffer for radioimmunoassay (RIA) was 50 mM sodium acetate buffer (pH 6.2) containing 0.1% BSA, 0.01% Triton X-100, 1 mM EDTA 2Na and 0.025% NaN_3. The RIA incubation mixture consisted of 50 µl of either the standard or the sample solution, 100 ul of antiserum at a dilution of 1 : 5000 and 50 µl of ^{125}I-labelled ligand (18,000 cpm) in the standard buffer. The above mixture was placed in a plastic tube (7.5 x 78 mm), mixed well, and then equilibrated at 4°C for 24 hr. The incubation was stopped by adding 50 µl of 1 % bovine γ-globulin and 500 µl of 25% polyethyleneglycol (#6,000) in the standard buffer. After vigorous shaking, the mixture was incubated at 4°C for 20 min. The mixture was centrifuged at 2,000 x g at 4°C for 30 min. The supernatant was aspirated, and radioactivity in the pellets was counted in a gamma counter. Assays were performed routinely in duplicate.

Preparation of Washed Platelets: Rat washed platelets used in this study were prepared as described (10), with some modification. Rats were anesthetized with an intraperitoneal injection of Nembutal (60 µg/g of body weight), and blood was drawn from the abdominal vein into a syringe containing 20 mg of EDTA in 3 ml of 0.9% NaCl. The blood was centrifuged at 100g for 20 minutes at room temperature to separate platelet rich plasma from erythrocytes. An equal volume of washing medium, consisting of 135 mM NaCl, 13 mM sodium citrate, 4 mM glucose, and 2 mM EDTA, pH 6.5, was added to the packed erythrocytes and the mixture was centrifuged again at 100g. The platelet rich plasma was then centrifuged at 800g for 10 minutes, and the platelet sediment was washed twice. The platelets were washed again with suspension medium, containing 135 mM NaCl, 2 mM EDTA, 5 mM glucose and 15 mM Hepes, pH 7.5, and resuspended in 1 ml of suspension medium. Platelets were counted by Coulter counter (Coulter Electronics Inc., Hialeah, Fl), and diluted to 1.6 x 10^5/µl for the following assay.

Assay Procedure: Twenty five microliters of sample dissolved in suspension medium containing 10 mM theophylline, was preincubated at 37°C for 10 min. The reaction was initiated by adding 25 µl of the above platelets and incubated for 30 seconds. The reaction was stopped with 200 µl of ethanol containing 150 mM HCl and heated for 3 min as previously described (10). The sample was evaporated in a speedvac concentrator and the extracts were dissolved in 100 µl of 50 mM sodium acetate buffer (pH 6.2). Cyclic AMP in the solution was succinylated by adding 30 µl of dioxan containing 650 mM succinic anhydrate and 10 µl of triethylamine. After standing at room

temperature for 30 min, the solution was lyophilized, dissolved in RIA buffer and analyzed by cAMP RIA.

Preparation of Peptide Extract: Human PC tissue (140g) was resected at surgery from a norepinephrine dominant PC patient. Diced pieces of the tumor were boiled for 10 min in 4 volumes of 1 M acetic acid to inactivate intrinsic proteases. After cooling, the mixture was homogenized at 4°C with a polytron mixer. The supernatant of the extracts, obtained after 30 min centrifugation at 20,000 x g, was subjected to acetone-precipitation at a concentration of 66%. After removal of the precipitates, the supernatant was concentrated by rotary evaporator. The concentrate was twice diluted with H_2O and applied on a C-18 silica gel column (270ml, Chemco LC-SORB ODS). The materials adsorbed on the column were eluted with 60% CH_3CN containing 0.1% trifluoroacetic acid (TFA). The eluates were evaporated and loaded onto a SP-Sephadex C-25 column (H^+-form, 2 x 15cm, pharmacia) in 1M acetic acid. Successive elution with 1M acetic acid, 2M pyridine, and 2M pyridine-acetate (pH 5.0) yielded three fractions: SP-I, SP-II and SP-III, respectively. The SP-III fraction, which contained about 90% of the activity, was used as the starting material in the present survey.

Structural Analysis: The purified peptides were sequenced with a gas phase sequencer, equipped with reverse phase HPLC for identification of the resulting phenylthiohydantoin (PTH) amino acids (Applied Biosystems, 470A/120A).

Results and Discussion

Radioimmunoassay for cAMP: The antiserum to succinyl (suc) cAMP recognized the suc cAMP with high affinity at a final dilution of 1:10,000. As shown in Fig. 1, the half maximal inhibition of radioiodinated ligand binding by suc cAMP was observed at 4 pg/tube. From 0.5 to 128 pg/tube of suc cAMP

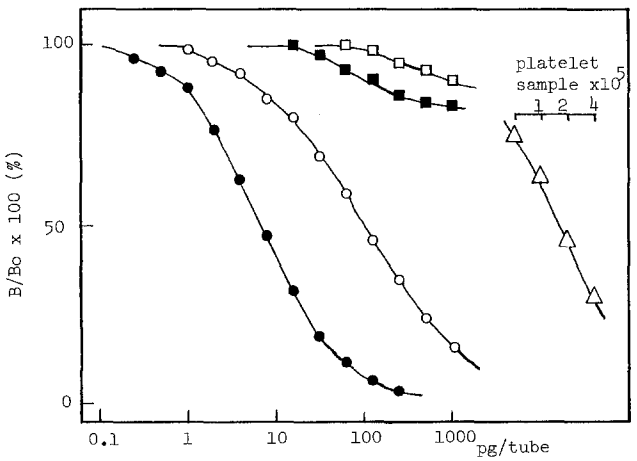

Fig. 1. Inhibition of binding of ^{125}I-succinyl cAMP to the antiserum against suc cAMP(final dilution 1:10000).
(-●-●-): suc cAMP. (-○-○-): cAMP.
(-■-■-): suc cGMP. (-□-□-): cGMP.
Inhibition of ^{125}I-suc cAMP binding to the antiserum by serial dilution of the platelet sample (-△-△-). The dilution curves are roughly parallel to those of standard suc cAMP.

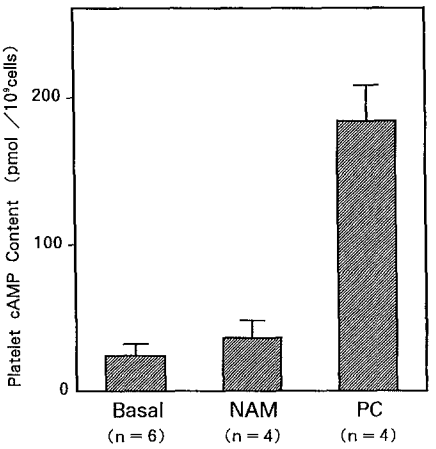

Fig. 2. Effect exposing platelets to peptide samples from normal adrenal medulla (NAM) and pheochromocytoma (PC). One hundred milligrams wet weight of peptide samples was reacted for 30 sec and the concentration of platelet cAMP was measured. Basal: without peptide sample. Data are shown as mean ± S. E. M.

was measurable by this RIA system. The intra- and inter assay coefficients of variance were less than 4 and 9%, respectively. This antiserum recognized suc cAMP with high affinity, had 6% crossreactivity with cAMP, and less than 0.1% crossreactivity with cGMP and suc cGMP as shown in Fig 1. Dilution of the platelet RIA sample, prepared as described in Methods, yielded competition curves that were roughly parallel to the standard curves of suc cAMP as shown in Fig. 1.

Comparison between normal adrenal medulla and pheochromocytoma: Normal adrenal medullae (n=4) were obtained from cadavers, and PC tissues (n=4) were obtained at surgery. Peptide samples (SP-II and SP-III) from each tissue were prepared as described Materials and Methods, and 0.1g wet weight of peptide sample was assayed. As shown in Fig. 2, peptide extract from normal adrenal medulla increased platelet cAMP 1.5-fold, however the peptide extract from PC increased it 5-fold. These data suggest that PC contained an abundant amount of peptides that elevate platelet cAMP. Although it has not hitherto been shown that these peptides are excreted from PC, PC tissue appears to produce a large amount of peptides that act on rat platelet.

Isolation and Characterization: About 90% of the activity which elevates platelet cAMP was found in SP-III, which included basic peptides and was prepared as described in Materials and Methods. Gel filtration of SP-III on Sephadex G-50 revealed that the activity was found in the Mr range 3000-4000 (data not shown). The fraction was lyophilized and the dry material (37mg) was dissolved in 10 mM $HCOONH_4$ (pH 6.5) containing 10 %

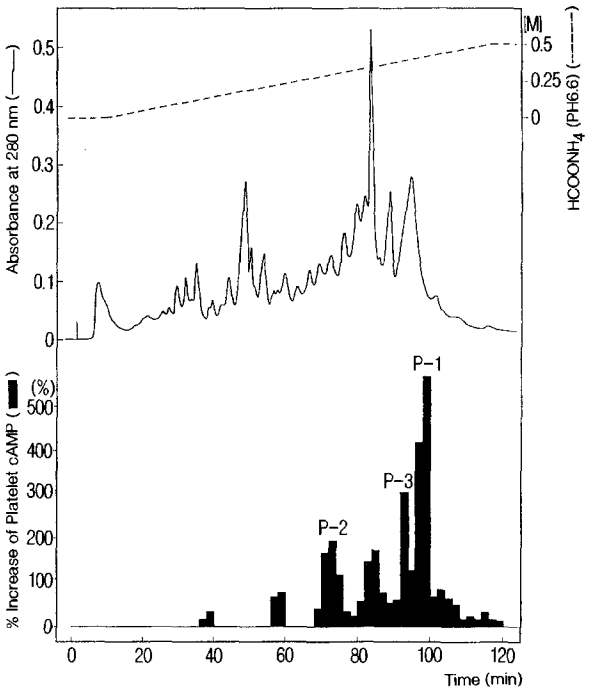

Fig. 3. Ion exchange HPLC of peptides which elevate platelet cAMP.
Sample: bioactive fraction after Sephadex G-50.
Column: TSK CM-2SW, 4.6 x 250 (mm). Flow rate: 1.0 ml/min.
Solvent system:
(a) 10 mM $HCOONH_4$ (pH 6.5) : CH_3CN = 90 : 10 (v/v)
(b) 1.0 M $HCOONH_4$ (pH 6.5) : CH_3CN = 90 : 10 (v/v).
Linear gradient elution from A : B = 100 : 0 to
A : B = 50 : 50 (120 min).
Major bioactive peaks were termed P-1, P-2 and P-3.

CH_3CN. The peptides were adsorbed on a columm of TSK IEX CM-2SW (8.0 x 300 mm Toyosoda) which had been equilibrated with 10 mM $HCOONH_4$ (pH 6.5) containing 10% CH_3CN. The column was washed with the same buffer and the peptide were eluted with a linear gradient of 10-500mM $HCOONH_4$. As shown in Fig. 3, three major peaks of activity and several minor peaks were observed. The three major peaks were termed P-1, P-2 and P-3 as shown in Fig. 3. Each peak was purified to homogeneity by three steps of reverse phase HPLC using Chemcosorb 7 ODS H (10.0 x 250 mm), Vydac phenyl (4.6 x 300 mm) and Micro Bondasphere ODS (4.6 x 150 mm) column. Figure 4A-C shows the final purification of peptides that elevate platelet cAMP from PC. The elution profiles of both A_{210} and the activity were in exact agreement, suggesting that peptides were homogenous. The recoveries of P-1, P-2 and P-3 were 4 μg, 1 μg and 0.2 μg, respectively, starting from 140g of PC tissue. The purified peptides were subjected to gas phase sequencing and the results are summarized in Table I. Up to the 28th amino acid (C-terminus) of P-1 was detectable. Up to the 26th and 23th amino acid was

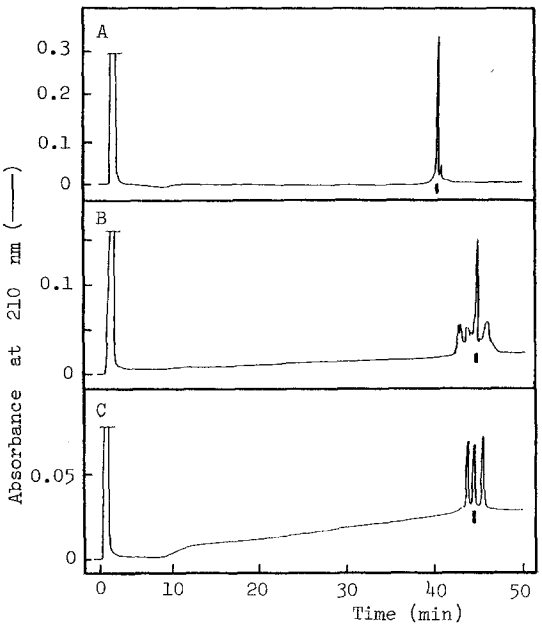

Fig. 4. Final purification of P-1 (A), P-2 (B), P-3 (C).
Each sample was obtained from P-1, P-2 and P-3 in Fig. 2.
Column: Micro Bondasphere ODS 300A (waters)
Solvent system: (A) H_2O : CH_3CN : 10% TFA = 90 : 10 : 1
(B) H_2O : CH_3CN : 10% TFA = 40 : 60 : 1.
Linear gradient elution from A : B = 20 : 80 to
A : B = 0 : 100 (80 min).
Black bars indicate the activity which elevates platelet cAMP.

detected by P-2 and P-3, but further amino acids sequence could not be determined because the sample amount was limited. The amino acid sequences of P-1, P-2 and P-3 were identical to those of vasoactive intestinal polypeptide (VIP), calcitonin gene related peptide (CGRP-I) and CGRP-II. To compare the retention time of each synthetic peptides in reverse phase HPLC as well as cation exchange HPLC, we concluded that P-1, P-2 and P-3 are VIP, CGRP-I and CGRP-II respectively. The other activity peaks described in Fig. 3 are now being purified. Human VIP (11), CGRP-I (12) and CGRP-II (13) are known biologically active peptides whose amino acid sequences have been deduced from that of the cDNA sequence complementary to the mRNA coding for each peptide. In the present study, these human peptides were originally isolated from PC tissue as endogenous peptides that elevate rat platelet cAMP. Therefore the present assay seems to be a useful tool for investigating biologically active peptides that act upon platelets.

Activity of VIP and CGRP-I: We examined the platelet cAMP elevating activities of synthetic VIP and CGRP-I. As shown in Fig.5, both peptides significantly increased rat platelet cAMP at low concentrations, and

Table I
Amino Acid Sequence Determination of P-1, P-2 and P-3

Number	P-1		P-2		P-3	
		pmol		pmol		pmol
1)	His	11.4	Ala	24.7	Ala	9.8
2)	Ser	7.7	X		X	
3)	Asp	27.5	Asp	18.2	Asn	9.1
4)	Ala	28.8	Thr	2.6	Thr	1.4
5)	Val	20.4	Ala	14.8	Ala	4.4
6)	Phe	27.9	Thr	3.0	Thr	1.1
7)	Thr	5.3	X		X	
8)	Asp	12.3	Val	6.2	Val	2.1
9)	Asn	18.4	Thr	1.1	Thr	0.5
10)	Tyr	11.0	His	3.0	His	1.4
11)	Thr	2.1	Arg	3.3	Arg	2.0
12)	Arg	3.5	Leu	5.0	Leu	1.1
13)	Leu	10.9	Ala	5.3	Ala	1.6
14)	Arg	4.2	Gly	4.5	Gly	1.1
15)	Lys	5.3	Leu	4.4	Leu	1.2
16)	Gln	6.7	Leu	6.7	Leu	2.1
17)	Met	3.5	Ser	0.8	Ser	0.6
18)	Ala	4.6	Arg	1.3	Arg	1.3
19)	Val	2.5	Ser	0.5	Ser	0.4
20)	Lys	1.8	Gly	0.9	Gly	1.4
21)	Lys	2.4	Gly	2.0	Gly	1.7
22)	Tyr	2.6	Val	0.5	Met	0.6
23)	Leu	2.3	Val	0.8	Val	0.4
24)	Asn	6.0	Lys	0.4		
25)	Ser	0.4	Asn	0.4		
26)	Ile	0.7	Asn	0.6		
27)	Leu	0.86				
28)	Asn	0.47				

X: Not detectable because of Cys.

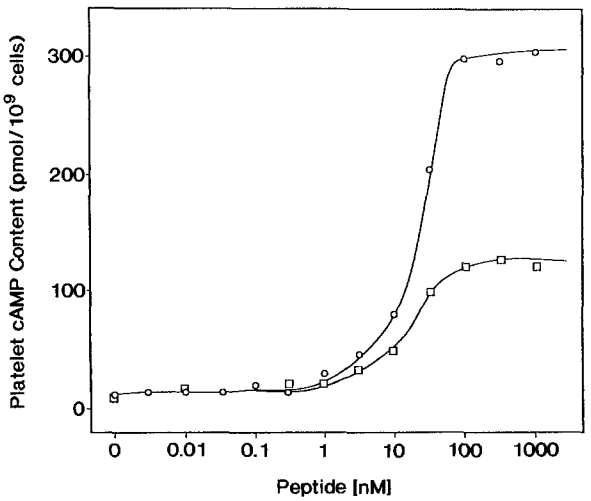

Fig. 5. Concentration of platelet cAMP in response to various doses of VIP (-O-O-) and human CGRP-I (-□-□-).
Half-maximal elevation of VIP and CGRP-I were observed at 32 and 14 nM, respectively.

showed a dose dependent increase of rat platelet cAMP. VIP and CGRP-I increased platelet cAMP levels 15- and 6-fold, respectively. The ED_{50} valeus of VIP and CGRP-I are 32 and 14 nM respectively. VIP and CGRPs are potent vasorelaxants, and increase blood vessel cAMP levels (14). In the present study, we purified VIP and CGRPs as an endogenous substance that elevates platelet cAMP and showed that they elicited a potent activity that elevates platelet cAMP. These data indicate the possibility that VIP and CGRPs act upon platelets as well as upon vascular tissue.

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