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response of canine coronary artery to isoproterenol and salbutamol after removal of endothelial cells.
Gen Pharmacol 1986; 17: 497—9.

7 Nakane T, Tsujimoto G, Hashimoto K, Chiba S. Beta adrenoceptors in the canine large coronary arteries: beta-1 adrenoceptors predominate in vasodilation.
J Pharmacol Exp Ther 1988; 245: 936—43.

8 Bernard F, Jouquey S, Hamon G. Study of the vasodilating activity of salbutamol on dog coronary arteries: Unexpected effects of methylene blue.
Pharmacology 1991; 42: 246—51.

9 Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 1980; 288: 373—6.

10 Chu A, Chambers DE, Lin C-C, Kuehl WD, Cobb FR. Nitric oxide modulates epicardial coronary basal vasomotor tone in awake dogs.
Am J Physiol 1990; 258: H1250—4.

血管内皮对狗离体冠状动脉 β肾上腺素受体激动剂反应性的影响

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目的: 研究内皮细胞对离体冠状动脉 β 肾上腺素受体激动剂反应性的影响. **方法**: 狗冠状动脉环离体实验, 生理记录仪记录血管张力. **结果**: 去甲肾上腺素(NE)和异丙肾上腺素(Iso)引起离体狗冠状动脉剂量依赖性舒张反应, 酚妥拉明加强 NE 的作用. 血管去内皮后, 对 NE 和 Iso 的反应减弱, 一氧化氮(NO)合成酶抑制剂 N^{ω}-硝基左旋精氨酸甲酯亦可减弱 NE 和 Iso 的作用. **结论**: β 肾上腺素受体激动剂对狗冠状动脉舒张作用部分依赖于内皮. 此作用由 NO 介导.

关键词 冠状血管; 血管内皮; 去甲肾上腺素; 异丙肾上腺素; 酚妥拉明

BIBLID: ISSN 0253-9756 Acta Pharmacologica Sinica 中国药理学报 1995 Jul; 16 (4): 360—365

Thrombin-induced neuropeptide Y secretion from rat platelets[1]

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AIM: To study the thrombin-induced secretion of platelet neuropeptide Y (NPY) in rats. **METHODS**: The platelet aggregation induced by ADP or thrombin was recorded by an aggregometer. NPY in platelet and plasma was measured by radioimmunoassay. The intracellular free Ca^{2+} ($[Ca^{2+}]_i$) was measured by Fura-2 fluorescent assay. **RESULTS**: Thrombin 0.75 or 2.5 kU L^{-1} increased $[Ca^{2+}]_i$ from 119±8 nmol L^{-1} to 530±60 or 1340±100 nmol L^{-1}, respectively together with the secretion of platelet NPY. Edetic acid 2 mmol L^{-1} almost abolished the thrombin-induced increases of $[Ca^{2+}]_i$ and reduced the NPY secretion by 56 % and 30 %, respectively. Neither $[Ca^{2+}]_i$ increase nor platelet NPY secretion induced by thrombin was affected by verapamil. The thrombin-induced NPY secretion was inhibited by 55 %—70 % by indometacin or creatine phosphate plus creatine phosphokinase. **CONCLUSION**: Thrombin-induced platelet NPY secretion was related to an Ca^{2+} influx

[1] Project supported by the National Natural Science Foundation of China. № 39270776 and the research grant from the State Educational Commission of China.
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Received 1994-05-23 Accepted 1994-11-29

through non-voltage dependent Ca^{2+} channels and positive feedbacks of arachidonate metabolites or/and released ADP.

KEY WORDS neuropeptide Y; blood platelets; platelet aggregation; thrombin; adenosine diphosphate; verapamil; edetic acid

Neuropeptide Y (NPY) widely distributed in the nervous system is usually co-stored and co-released with norepinephrine (NE) in sympathetic nerve endings[1]. Recently, immunoreactive NPY was also synthesized in rat megakaryocytes and stored in platelets[2]. The stored NPY in platelets was secreted during platelet activation[3]. We have observed that NPY secreted from platelets enhanced the vasoconstriction induced by co-released substances from platelets[4]. NPY secreted from platelets in some pathophysiological states, along with NPY secreted from sympathetic nerve endings may be involved in some cardiovascular diseases[5]. Changes in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) form an important part of the stimulus-response coupling pathway in platelets[6]. The formation of arachidonate metabolites and released adenosine diphosphate (ADP) during platelet activation also play an important role in the regulation of platelet functions[7]. The aim of this study was to determine the role of $[Ca^{2+}]_i$, arachidonate metabolites and released ADP in the thrombin-induced NPY secretion in rat platelets.

In our preliminary experiments, calcium antagonist nifedipine inhibited the thrombin-induced platelet aggregation and NPY secretion.

MATERIALS AND METHODS

Drugs ADP, indomethacin (Ind), verapamil (Ver), nifedipine (Nif), Fura 2-AM, bovine serum albumin (BSA), egtazic acid, edetic acid, phosphocreatine (PC), and creatine kinase (CK) (Sigma, St Louis MO, USA); thrombin (Beijing Biochemical Products Co); porcine NPY (Peninsula Laboratories Inc. USA); [^{125}I] NPY (Amersham, Arlington Heights IL, USA). Anti-NPY antiserum was a gift from Prof JK McDonald (Emory University, USA).

Isolation of platelet-rich-plasma (PRP) and platelets Wistar rats (♂, 250±s 50 g) were anesthetized with urathane (1 g kg^{-1}, ip) and blood was collected from the abdominal aorta into tubes containing 3.8 % sodium citrate (1:9 vol). Citrated blood was centrifuged at 100×g for 15 min and the supernatant was collected as PRP. The PRP was centrifuged at 1 200×g for 10 min to obtain the supernatant as platelet-poor-plasma (PPP). The platelet pellets were washed once by buffer containing NaCl 123, glucose 30, sodium citrate 13 mmol L^{-1}, pH 6.8, and resuspended in Tyrode buffer containing NaCl 140, KCl 3, $MgSO_4$ 1, HEPES 10, glucose 10 mmol L^{-1}, pH 7.4 adjusted by NaOH 5 mmol L^{-1}. Platelet number in PRP or the washed platelets (WP) was counted in phase contrast microscopy and was adjusted with PRP or Tyrode buffer to 10^{12} platelets L^{-1}. Except for the measurement of $[Ca^{2+}]_i$, PRP, and WP were kept in an ice bath before use.

Platelet aggregation Platelet aggregation was studied in an aggregometer[8] (LM 14—104 Da Hua Instrument Co, Shanghai). Samples were equilibrated at 37 C for 2.5 min at continuous stirring (1000 rpm). After addition of ADP to PRP or thrombin to WP, aggregation curves were recorded for 4 min and analyzed in terms of % of maximal increase in light transmittance. Then PRP or WP was centrifuged at 2200×g for 15 min, and the supernatant was stored at −30 C for NPY determination.

Each inhibitor was added for 3 min, except for indometacin (5 min) and CP/CPK (0.5 min), prior to addition of agonists. The concentration of Ca^{2+} in WP was adjusted by addition of Ca^{2+} 1 mmol L^{-1} or edetic acid 2 mmol L^{-1}.

Determination of NPY-immunoactivity After extraction of samples with cold acid-ethanol, NPY was measured by radioimmunoassay with [^{125}I]NPY as the tracer[4]. The NPY antiserum used has negligible cross reaction to peptides of similar size and structure. In addition to measurement in post-aggregation supernatant, the NPY was quantified in PRP or WP which

was not aggregated, but in which platelets were disrupted during the extraction procedure. NPY-immunoactivity was also measured in PPP and plasma prepared from whole blood.

Characterization of NPY from platelets by reverse-phase HPLC WP was stimulated maximally with thrombin to induce aggregation. After rapid centrifugation, the supernatant was extracted with ethanol, filtered, injected onto a C_{18} 5 μm, 4.6 mm × 150 mm column (Beckman Instrutments, USA) and eluted with a 60-min linear gradient of 1 % to 60 % acetonitrile containing 0.1 % trifluoroacetic acid[12]. Fractions of 1 mL were collected at an elution rate of 1.0 mL min^{-1} and assayed for NPY. Porcine NPY standards and WP were run simultaneously.

Measurement of $[Ca^{2+}]_i$ in platelets Washed platelets were prepared as above and loaded with Fura 2-AM 5 μmol L^{-1} in Tyrode buffer containing 5 % (wt/vol) BSA at 37 ℃ for 45—60 min. The platelet pellet was resuspended in Tyrode buffer containing 0.05 % BSA and stored at 20 ℃. Prior to the fluorescence measurement, aliquots of platelets (2 mL) were equilibrated at 37 ℃ for 3 min in the presence or absence of inhibitors. The concentration of Ca^{2+} in WP was adjusted by adding Ca^{2+} 1 mmol L^{-1} or edetic acid 2 mmol L^{-1} as required.

Fluorescence of platelet suspensions containing about 2×10^{11} platelets L^{-1} was measured with a RF 5000 spectrophotometer (Shimadzu Co, Japan). The emission wavelength was 500 nm and the excitation wavelength was switched continuously between 340 nm and 380 nm. Cytoplasmic Ca^{2+} concentration was calculated[13]: $Ca^{2+} = K_d(R - R_{min})/(R_{max} - R) \times (F_o/F_s)_{380}$, where R_{max}, R_{min}, and $(F_o/F_s)_{380}$ were determined by the addition of 0.1 % (wt/vol) Triton X-100 in the presence of $CaCl_2$ 1 mmol L^{-1} or egtazic acid 10 mmol L^{-1} and Tris 50 mmol L^{-1} (pH 8.3). To check the leakage of Fura 2, $MnCl_2$ 1 mmol L^{-1} was added[14]. Dye leakage proved to be minor and was not taken into consideration in the calibration procedure.

Statistics All results are expressed as $\bar{x} \pm s$. The t test was used for comparison of two groups.

RESULTS

High levels of NPY in PRP and WP

NPY was present at high levels in PRP and WP (59±8 μg L^{-1} and 49±6 ng/10^9 platelets, respectively, $P>0.05$, $n=9$), but was much lower in PPP and plasma (3.4 ± 0.4 and 3.9±0.7 μg L^{-1}, respectively, $n=9$).

Platelet aggregation and NPY secretion

Stimulation of platelets with thrombin resulted in a second irreversible aggregation paralleled with NPY secretion. When ADP was used as a stimulus, only a primary reversible aggregation occurred and a minor NPY secretion was produced (Fig 1).

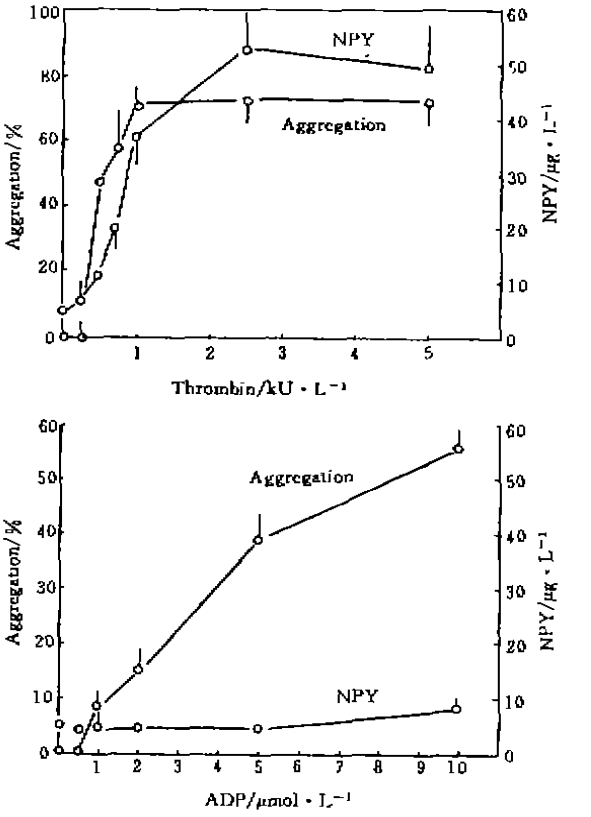

Fig 1. Platelet aggregation and NPY secretion induced by thrombin in washed platelets ($n=6$) or by ADP in PRP ($n=8$).

Characterization of NPY from platelets

The elution profile of NPY in post-aggregation supernatant and WP consisted of a single immunoreactive peak at a position corresponding to authentic porcine NPY.

Effects of external Ca^{2+} on platelet aggregation and NPY secretion In the presence of edetic acid 2 mmol L^{-1} in PRP or WP, the platelet aggregation induced by thrombin 0.75 kU L^{-1} was abolished (only a change in shape), and the secondary aggregation induced by thrombin 2.5 kU L^{-1} was completely inhibited. On the other hand, the NPY secretion induced by thrombin 0.75 and 2.5 kU L^{-1} was only inhibited by 56 % and 30 %, respectively (Tab 1).

Effect of Ver on platelet aggregation and NPY secretion In the presence of the Ver 5—50 μmol L^{-1}, neither platelet aggregation nor NPY secretion stimulated with thrombin (0.75 or 2.5 kU L^{-1}) was affected. Only at an extremely high concentration of Ver 500 μmol L^{-1}, platelet aggregation induced by thrombin 0.75 kU L^{-1} was inhibited by 50 %, and the NPY secretion was still unchanged (Tab 1).

Measurement of $[Ca^{2+}]_i$ in Fura 2-loaded platelets In the presence of $CaCl_2$ 1 mmol L^{-1}, thrombin 0.75 or 2.5 kU L^{-1} increased $[Ca^{2+}]_i$ from the resting level of 119±8 nmol L^{-1} ($n=8$) to 530±60 nmol L^{-1} ($n=4$) or 1340±100 nmol L^{-1} ($n=7$), respectively. Ver 0.1 mmol L^{-1} did not affect the $[Ca^{2+}]_i$ increase induced by thrombin 2.5 kU L^{-1} (1430±90 nmol L^{-1} *vs* 1430±140 nmol L^{-1}, $n=4$, $P>0.05$).

In the calcium-free edetic acid 2 mmol L^{-1} medium, thrombin 2.5 kU L^{-1} resulted in only a slight and transient increase of $[Ca^{2+}]_i$ from the resting level of 80±4 nmol L^{-1} to 150±60 nmol L^{-1} ($n=8$, $P<0.05$). After $[Ca^{2+}]_i$ returned to the original baseline level, a rapid increase of Ca^{2+} in the medium to about 1 mmol L^{-1} induced a second peak in $[Ca^{2+}]_i$ at 1340±50 nmol L^{-1} ($n=4$) in platelets, which were indistinguishable from the calcium signal in the control experiments.

Effect of Ind, PC/CK on platelet aggregation and NPY secretion When thrombin 0.75 kU L^{-1} was used as a stimulus, platelet aggregation was completely inhibited by either Ind 20 μmol L^{-1} or PC 5 mmol L^{-1} plus CK 100 kU L^{-1}, and its NPY secretion was inhibited by 70 % and 55 %, respectively. When thrombin 2.5 kU L^{-1} was used, both aggregation and NPY secretion were inhibited by 60 %—70 % by either Ind or PC/CK. Ind and PC/CK used together abolished aggregation and inhibited NPY secretion induced by thrombin 2.5 kU L^{-1} by 82 % (Tab 2).

Tab 1. Platelet aggregation and NPY secretion induced by thrombin in edetic acid or verapamil. $^bP<0.05$ ***vs* control.**

		Thrombin 0.75 kU L^{-1}		Thrombin 2.5 kU L^{-1}	
		Aggregation/ %	NPY/ μg L^{-1}	Aggregation/ %	NPY/ μg L^{-1}
Verapamil/ μmol L^{-1}	0	0.2±0	3.5±0.4	0.6±0	3.1±0.4
	Control	50±4	42±6	68±3	64±10
	5	56±5	32±6	71±2.5	69±2.0
	50	52±6	33±7	70±1.1	58±1.9
	500	25±8	43±6	62±10	69±12
Edetic acid/ mmol L^{-1}	2	0.7±0^b	21±8^b	13±5^b	41±8^b

Tab 2. Effects of inhibitors on aggregation and NPY secretion induced by thrombin in washed platelets. Ind 20 μmol L^{-1}; PC 5 mmol L^{-1}; CK 100 kU L^{-1}. $^bP<0.05$ *vs* control; $^cP<0.05$ *vs* Ind or PC/CK.

	Thrombin 0.75 kU L^{-1}		Thrombin 2.5 kU L^{-1}	
	Aggregation/ %	NPY/ μg L^{-1}	Aggregation/ %	NPY/ μg L^{-1}
Control	51±4	41±6	68±3^b	64±11^b
Ind	0.6±0	13±6^b	20±7^b	22±7^b
PC/CK	0.6±0	17±7^b	22±7^b	18±4^b
Ind/PC/CK	—	—	0.4±0	7.9±0.5^{bc}

DISCUSSION

This paper showed that stimulation of rat platelet with thrombin resulted in NPY secretion parallel to its aggregation. In contrast, when ADP was used as the stimulus, no NPY was secreted, although a concentration-dependent aggregation was produced. This indicates that NPY is secreted during platelet activation evoked by some agonists, but NPY secretion is not necessarily parallel to platelet aggregation.

It is generally accepted that a rise in cytosolic Ca^{2+} concentration was the final common pathway for platelet activation. Using Fura 2 as an indicator, we found that thrombin could induce an increase of $[Ca^{2+}]_i$ in platelets, and chelation of external Ca^{2+} markedly reduced the increase of $[Ca^{2+}]_i$ evoked by thrombin and accelerated the return of $[Ca^{2+}]_i$ to the resting level. Consistently, the second aggregation was abolished and NPY secretion was partly inhibited after chelation of external Ca^{2+}. It suggests that the increase of $[Ca^{2+}]_i$ is mainly due to influx of external Ca^{2+} and may be one of the mechanisms for thrombin-induced platelet aggregation and NPY secretion. Furthermore, the different effects of external Ca^{2+} chelation on platelet aggregation and NPY secretion seen in our experiments indicate that the mechanisms involved in the two processes are not the same. The results showed that clinical pharmacological concentrations of Ver 5—50 μmol L^{-1} had no effects on thrombin-induced platelet aggregation and NPY secretion. Even very high concentration of Ver 500 μmol L^{-1} still did not affect thrombin-induced NPY secretion. Consistently, Ver 0.1 mmol L^{-1} did not affect thrombin-induced Ca^{2+} influx, as indicated by the increase of $[Ca^{2+}]_i$ measured with Fura 2. Our preliminary experiments showed that Nif also had not significant effects on platelet aggregation, NPY secretion and Ca^{2+} influx. These results suggest that voltage-dependent Ca^{2+} channels do not exist or at least do not play an important role in rat platelets. The inhibitory effect of Nif on thrombin-induced platelet aggregation at a very high concentration (0.5 mmol L^{-1}) is probably due to its nonspecific action on platelet function[11].

The present study also shows that Ind, which inhibited cyclo-oxygenase and abolished the formation of arachidonate metabolites, and PC/CK which convert, released ADP to ATP greatly inhibited thrombin-induced platelet aggregation and NPY secretion. The effects of Ind and PC/CK could be additive. These results suggests that arachidonate metabolites and released ADP are two major positive feedback factors to potentiate thrombin-induced platelet aggregation and NPY secretion. The mechanisms of the positive feedback action

need to be determined.

In conclusion, thrombin-induced NPY secretion from platelets was related to a Ca^{2+} influx through non-voltage dependent Ca^{2+} channels and a positive feedback of arachidonate metabolites or/and released ADP.

REFERENCES

1 Ekblad E, Edvinsson L, Wahlestedt C, Uddman R, Hakanson R, Sundler F. Neuropeptide Y co-exists and co-operates with noradrenaline in perivascular nerve fibers. Regul Pept 1984; **8**: 225—35.

2 Ericsson A, Hemsen A, Lundberg JM, Persson H. Detection of neuropeptide Y-like immunoreactivity and messenger RNA in rat platelets; the effects of vinblastine, reserpine, and dexamethasone on NPY expression in blood cells. Exp Cell Res 1991; **192**: 604—11.

3 Myers Ak, Farhat MY, Vaz CA, Keiser HR, Zukowska-Grojec Z. Release of immunoreactive-neuropeptide Y by rat platelets. Biochem Biophys Res Commun 1988; **155**: 118—22.

4 Yang YF, Yang LH, Shi GP, Han QD. Platelet neuropetide Y and its vasoconstriction effect in rat. Acta Physiol Sin 1993; **45**: 400—4.

5 Ogawa T, Kitamura K, Kangawa K, Matsuo H, Eto T. Platelet neuropeptide Y in spontaneously hypertensive rats. J Hypertens 1992; **10**: 765—71.

6 Rink TJ, Sage SO. Calcium signaling in human platelets. Annu Rev Physiol 1990; **52**: 431—49.

7 Holmsen H. Prostaglandin endoperoxide-thromboxane synthesis and dense granule secretion as positive feedback loops in the propagation of platelet responses during "the basic platelet reaction." Thromb Haemost 1977; **38**: 1030—41.

8 Born GVR. Aggregation of blood platelets by adenosine diphosphate and its reversal. Nature 1962; **194**: 927—9.

9 Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 1985; **260**: 3440—50.

10 Hallam TJ, Rink TJ. Agonists stimulate divalent cation channels in the plasma membrane of human platelets. FEBS Lett 1985; **186**: 175—9.

11 Bonadonna G, Lechi C, Corradini P, Sinigaglia D, De Togni P, Miroslawa G. Verapamil inhibits platelet aggregation by a calcium independent mechanism. Thromb Haemost 1986; **56**: 308—10.

凝血酶引起大鼠血小板神经肽 Y 释放的机制

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目的：研究凝血酶引起血小板释放 NPY 的作用及其机制。**方法**：用血小板聚集仪记录凝血酶及 ADP 引起的血小板聚集；用放射免疫法测定血小板及血浆中的 NPY；用 Fura 2荧光测定法记录细胞内游离 Ca^{2+}（$[Ca^{2+}]_i$）。**结果**：凝血酶在引起 NPY 释放的同时，细胞内游离钙（$[Ca^{2+}]_i$）升高。依地酸基本消除凝血酶引起的 $[Ca^{2+}]_i$ 增高，并显著减少凝血酶引起的 NPY 释放。维拉帕米对凝血酶的作用无显著影响。磷酸肌酸与磷酸肌酸激酶合用或消炎痛均可使凝血酶引起的 NPY 释放减少。**结论**：凝血酶引起的血小板 NPY 释放与(1)细胞外 Ca^{2+} 通过非电压依赖性钙通道进入及(2)花生四烯酸代谢物和 ADP 释放的正反馈机制有关。

关键词 神经肽 Y；血小板；血小板聚集；凝血酶；腺苷二磷酸；维拉帕米；依地酸