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Coronary Vasoconstrictive Effects of Neuropeptide Y and Their Modulation by the ATP-Sensitive Potassium Channel in Anesthetized Dogs

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Objectives. **This study examined the coronary vasoconstrictive action of endogenous neuropeptide Y (NPY) during sympathetic nerve stimulation and its modulation by the adenosine triphosphate (ATP)–sensitive potassium (K_{ATP}) channel in vivo.**

Background. **Exogenous NPY was characterized by its potent vasoconstrictive effect. However, endogenous NPY has failed to show any vasoconstrictive activity in vivo.**

Methods. **We studied 70 anesthetized dogs with vagotomy under beta-adrenergic blockade. Ansae subclaviae stimulation and intracoronary administration of the neurotransmitters (NPY and norepinephrine) were done with or without alpha-adrenergic blockade, NPY antagonist BIBP3226 or K_{ATP} channel acting agents. We measured coronary vascular resistance (CVR) and the neurotransmitter levels in systemic arteries and the great cardiac vein, and the amount of overflow (venoarterial difference times myocardial blood flow).**

Results. **During nerve stimulation, NPY levels correlated significantly with CVR at the highest r value ($r = 0.850$, $p < 0.0001$) obtained for the venous level under alpha-blockade, but norepinephrine showed no correlation. Treatment with BIBP3226 abolished the correlation between NPY level and CVR under alpha-blockade. Without alpha-blockade, norepinephrine levels correlated significantly with CVR; however, NPY showed no correlation. The amount of NPY overflow during the stimulation was nearly 1,000-fold lower than norepinephrine overflow. Exogenous NPY had a 100-fold more potent coronary vasoconstrictive action than that of norepinephrine. The K_{ATP} channel antagonist glibenclamide enhanced vasoconstriction of NPY, and the agonist pinacidil suppressed it with a predominant effect in the subepicardial region.**

Conclusions. **During sympathetic nerve stimulation, the vasoconstrictive actions of NPY are masked by norepinephrine under intact alpha-adrenoceptor conditions, manifest during alpha-blockade and modulated by K_{ATP} channel activity.**

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Neuropeptide Y (NPY), a 36-amino acid peptide, has a wide distribution in both the central and the peripheral nervous system (1), and cardiac perivascular sympathetic nerve fibers contain high concentrations of NPY (2,3). In peripheral sympathetic nerves, NPY is cosecreted with norepinephrine (4). Exogenous NPY has been reported to have a variety of functions, including regulation of coronary blood flow, cardiac contractility and heart rate in anesthetized dogs (5–9). The NPY-induced vasoconstriction is mainly mediated by way of the Y_1-receptor (10), which was antagonized by BIBP3226 (11,12). The effect of endogenous NPY on the coronary circulation has not been determined. Otani et al. (13) concluded that the quantity of released NPY during sympathetic nerve stimulation was insufficient to elicit a coronary vasoconstriction in vivo. The major aim of the present study was to demonstrate endogenous NPY-related vasoconstriction during sympathetic nerve stimulation in vivo by examining the quantitative relation between both the endogenous level and the exogenous dose of NPY and changes in coronary vascular resistance (CVR).

Reactive hyperemia has been shown to be modulated by adenosine triphosphate (ATP)-sensitive potassium (K_{ATP}) channels during coronary occlusion (14). Recently, it has been demonstrated that K_{ATP} channels modify the reactivity of coronary vessels to exogenous norepinephrine (15,16). Therefore, the second aim of this investigation was to determine the effects of K_{ATP} channel activity on the relation between NPY and CVR.

Methods

General experimental conditions. This investigation conformed with the Guide for the Care and Use of Laboratory

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Abbreviations and Acronyms

ANCOVA	=	analysis of covariance
ANOVA	=	analysis of variance
ATP	=	adenosine triphosphate
CVR	=	coronary vascular resistance
%CVR	=	percent changes in coronary vascular resistance
K_{ATP} channel	=	adenosine triphosphate–sensitive potassium channel
NPY	=	neuropeptide Y

Animals published by the U.S. National Institutes of Health (NIH publication no. 86-23, revised 1985). Seventy male and female adult dogs (mean weight 14.3 ± 5.8 kg) were anesthetized with morphine hydrochloride and alpha-chloralose and artificially ventilated as described previously (15). We isolated the vagal nerves at the cervical level, severed them bilaterally and blocked beta-adrenoceptor activity by intravenous administration of propranolol (2 mg/kg body weight) 15 min before the initiation of the protocols to be described. We reinjected propranolol (0.5 mg/kg) every hour to maintain the beta-blockade to attenuate metabolic coronary regulation related to heart rate. In 38 of 70 dogs, phenoxybenzamine (1 mg/kg intravenously) was also administered to attenuate the vasoconstrictive effects of norepinephrine by way of the alpha-adrenoceptor.

In preliminary experiments, we investigated the effects of competitive blockade of the beta-adrenoceptor with propranolol (2 mg/kg intravenously) and the alpha-adrenoceptor with phenoxybenzamine (1 mg/kg, intravenously) by intravenous administration of isoproterenol (0.01 to 10.0 μg/kg per min) and intracoronary administration of phenylephrine (0.01 to 10.0 μg/kg per min), respectively. Propranolol elevated the threshold of isoproterenol on chronotropic action from 0.03 μg/kg per min to 3.0 μg/kg per min; phenoxybenzamine elevated the threshold of phenylephrine on CVR increase from 0.3 μg/kg per min to >10.0 μg/kg per min, although this dose caused an increase in aortic pressure >10%.

Specific surgical procedures and experimental protocols. *Protocol 1. Correlation between CVR and endogenous NPY and norepinephrine.* In 21 dogs, we performed sympathetic nerve stimulation and analyzed the correlation between CVR and the levels or the overflow of neurotransmitters (NPY and norepinephrine) including data pairs at rest, 2 Hz and 20 Hz under alpha- and beta-blockade (protocol 1A, 7 dogs), under beta-blockade (protocol 1B, 7 dogs) and under alpha- and beta-blockade plus NPY Y_1–selective antagonist BIBP3226 (1 mg/kg, intravenously, Dr. Karl Thomae GmbH, Biberach, Germany [protocol 1C, 7 dogs]).

Bilateral thoracotomy was performed in the fifth intercostal space; the sternum was separated horizontally and the pericardium incised. A 3F catheter was placed in the left atrium by way of an appendage for the injection of nonradioactive microspheres, and a 5F catheter was placed in the descending aorta to obtain reference blood samples for the measurement of myocardial blood flow. Two 7F catheters were placed in the aortic arch by way of the left carotid artery and in the great cardiac vein by way of the right jugular vein to obtain arterial and venous blood samples, respectively, for the measurement of neurotransmitter concentrations of NPY and norepinephrine. Aortic pressure was also recorded by means of the catheter in the aorta.

The bilateral ansae subclaviae, which branch off from the bilateral stellate ganglia, were isolated and ligated at the proximal sites. Two coated stainless steel bipolar electrodes were set on both sides of these nerves. The bilateral ansae subclaviae were electrically stimulated for 2.5 min at 2 Hz (5 V, 1 ms), followed by 20 Hz for 2.5 min. Arterial and venous blood samples were taken from the two 7F catheters during the final 1 min of each nerve stimulation period. In the same period, microspheres containing solution were injected to measure the coronary blood flow.

We calculated the correlation coefficient (r), determinant coefficient (r^2) and SD of the dependent variable from the regression line corrected to the mean value of the dependent variables ($Sy{\cdot}x/\bar{y}$) for correlation analysis.

Protocol 2. Role of the K_{ATP} channel in endogenous NPY stimulation of coronary vasoconstriction. In 14 dogs, we investigated the role of the K_{ATP} channel on coronary vasoconstriction by endogenous NPY under alpha- and beta-blockade. The experimental conditions were identical to those of protocol 1A except for placement of an additional 3F catheter into the left atrium. While either glibenclamide (0.6 mg/kg per min, Sigma Chemical, seven dogs) or pinacidil (10 μg/kg per min, Shionogi, Japan, seven dogs) was injected into the left atrium, the nerve stimulation and the analyses were performed according to protocol 1. Drug administration was started 2 min before the electrical stimulation.

Protocol 3. Reactivity of coronary vessels to exogenous NPY and norepinephrine. In 30 dogs, the changes in CVR induced by intracoronary administration of NPY and norepinephrine were examined. In the 10 dogs of protocol 3A, the dose response of CVR to NPY (5 dogs) was compared with the dose response to norepinephrine (5 dogs) under alpha- and beta-adrenoceptor blockade. In the 20 dogs of protocol 3B, the dose response of CVR to NPY (10 dogs) was compared with the dose response to norepinephrine (10 dogs) under beta-blockade.

Left-sided thoracotomy was performed at the fifth intercostal space, and immediately after the ligation of the branch a silicone tube bypass equipped with the flow probe of an electromagnetic flowmeter (MFV2100, Nihon Kohden, Japan) was set between the left subclavian artery and the proximal branch of the left circumflex coronary artery. Before placement of the bypass, heparin (3,000 to 5,000 U intravenously followed by 1,000 to 2,000 U/h) was administered. Coronary perfusion pressure was continuously recorded through the side path of a bypass circuit. A 7F catheter was placed in the aortic arch by way of the left carotid artery to measure the arterial pressure. We confirmed reactive hyperemia after a 15-s occlusion to be >200% of the basal flow 10 min before each experiment.

Either NPY (0.001, 0.003, 0.01, 0.03, 0.1, 0.3 μg/kg per min [i.e., 0.00023 to 0.07 nmol/kg per min]; synthetic human NPY, Peptide Institute, Japan) or norepinephrine (0.001, 0.003, 0.01, 0.03, 0.1, 0.3 μg/kg per min [i.e., 0.006 to 1.8 nmol/kg per min]; Waco Chemical, Japan) was administered through the side path of the bypass circuit into the coronary artery and the dose responsiveness of percent changes in coronary vascular resistance (%CVR) to NPY or norepinephrine was analyzed. The doses were increased in a stepwise manner every 1 min. In the last stage of the experiment, Evans blue solution was administered through the bypass to identify the area perfused by the bypass circuit.

We compared the dose response curves of %CVR to NPY and norepinephrine under alpha-blockade (protocol 3A) and under intact alpha-adrenoceptor (protocol 3B) by analysis of covariance (ANCOVA).

Protocol 4. Role of K_{ATP} channel in exogenous NPY stimulation of coronary vasoconstriction. In protocol 4A, we tested whether either of three K_{ATP} channel acting agents could modify the dose response to NPY under beta-blockade. Protocol 4A was performed in the 10 dogs in which the NPY dose response (protocol 3B) had been evaluated. After 30 min of protocol 3B, we confirmed recovery of heart rate and aortic pressure to 90% to 110% of the baseline value of protocol 3B. We then confirmed that the degree of the reactive hyperemia was not different from that of the reactive hyperemia just before protocol 3B (>200% of the basal flow). The dose response to NPY was determined again under intracoronary administration of a K_{ATP} channel antagonist (four dogs with glibenclamide [30 μg/kg per min]) or agonist (three dogs with pinacidil [0.5 μg/kg per min] and three dogs with nicorandil [10 μg/kg per min], Chugai Pharmaceutical, Japan). We compared the dose-response curves of %CVR to NPY with and without K_{ATP} channel-acting substances (the data for the control curves came from protocol 3B) by ANCOVA.

In protocol 4B, we analyzed the dependence of exogenous NPY and norepinephrine vasoconstriction on K_{ATP} channels in the other five dogs. Four dose-response trials were performed for each dog in the following order: 1) norepinephrine dose response, 2) norepinephrine dose response with nicorandil, 3) NPY dose response, and 4) NPY dose response with nicorandil. Between each trial we left a 30-min intermission. Before each trial, recovery of heart rate and aortic pressure to 90% to 110% of the original baseline value was confirmed. Then the reactive hyperemia was confirmed to be >200% of the basal flow. The substances tested above were administered into the coronary artery as in protocol 4A under beta-blockade. The degree of suppression by nicorandil in norepinephrine dose response was estimated by the difference in %CVR between the first and the second trials, and that in NPY dose response was between the third and the fourth trials. The effect of nicorandil suppression on NPY and norepinephrine was compared by ANCOVA.

Calculation of CVR. CVR (mm Hg/ml per min per 100 g) was calculated as mean coronary perfusion pressure (or mean aortic pressure) divided by coronary blood flow per 100 g of myocardium measured with nonradioactive microspheres of 15-μm diameter by the X-ray fluorescence method (17) (protocols 1 and 2) or with an electromagnetic flowmeter set at the subclavian-coronary bypass (protocols 3 and 4). The microspheres were labeled with one of nine sets of stable heavy elements (Ti, Br, Y, Zr, Nb, In, I, Ba, or Ce, Sekisui Plastic, Japan) and were suspended in 0.05% sodium dodecyl sulfate at a concentration of 2×10^6/ml. The microsphere solution (1 ml) was gently infused over a period of 30 s to 1 min, and reference blood was taken at a rate of 6 ml/min for 2 min beginning 10 s before the microsphere infusion. After completion of the experimental protocol, the heart was excised and the free wall of the left ventricle taken out. The myocardium was cut into two to three layers from the epicardium to the endocardium. The X-ray fluorescence of each heavy element was measured with a wave-length dispersive X-ray fluorescence spectrometer (PW1480, Phillips, Holland). The blood flow was calculated with the following equation:

Myocardial blood flow (ml/min per 100g)

= Sampling rate of reference blood × Tissue count

× 100/(Reference blood count × Tissue weight).

Measurement of NPY and norepinephrine. Blood (~3 ml) was collected in a chilled polystyrene tube, gently mixed with 20 mg of EDTA disodium, kept on ice for 5 min and then centrifuged at 1,500 g for 20 min at 4°C. Plasma was pipetted off and stored at −25°C until assay. NPY concentration was estimated by radioimmunoassay. To remove plasma protein, 1 ml of acid ethanol was added to 0.5 ml of plasma. After centrifugation, the supernatants were evaporated to dryness (18). Radioimmunoassay was performed according to the manufacturer's protocol (Amersham International plc, UK) using ^{125}I-labeled human NPY (74 TBq/mmol, Amersham). Synthetic human NPY was used as a standard. Anti-NPY rabbit serum (Peptide Institute) was diluted to a final concentration of 1:800,000. The antiserum revealed the specificity of human NPY and porcine NPY to be 100%; human 1-9NPY, human 24-36NPY, human pancreatic polypeptide and peptide YY were present at <0.1%. The detectable limit of NPY was 10 pmol/liter. The norepinephrine concentration was measured by fluorometric determination with diphenylethylenediamine using high-performance liquid chromatography (19) after removing plasma protein with perchloric acid. The detectable limit of norepinephrine was 0.03 nmol/liter. The overflows of NPY and norepinephrine in protocols 1 and 2 were calculated with the following equation:

Overflow of NPY or norepinephrine (mol/min per 100 g)

= (Concentration in the great cardiac vein

− Concentration in the aorta) × Coronary blood flow.

Statistical analysis. All data obtained in this study are expressed as mean value ± SD. We applied an analysis of variance (ANOVA) for repeated measures with the Scheffé F test to determine the significance of the hemodynamic changes

from the baseline during stimulation in all protocols, unpaired *t* test for the mean values of CVR and concentrations of the neurotransmitter between the two groups in protocol 2, and correlation analysis between the neurotransmitter and CVR in protocols 1 and 2. Analysis of covariance (ANCOVA) was performed in protocols 3, 4A and 4B to compare the dose responsiveness of the coronary vasculature to neurotransmitter administration. In all three protocols, the concentration of neurotransmitter was used as covariate. In protocols 3 and 4A, %CVR was used as the dependent variable and the degree of suppression of %CVR was the dependent variable in protocol 4B. In protocols 3 and 4B two kinds of neurotransmitters (NPY and norepinephrine) were used as the grouping variable, and the presence of K_{ATP}-acting agents was used in protocol 4A. Differences were considered significant where $p < 0.05$.

Results

Correlation between CVR and endogenous NPY (protocol 1). Bilateral ansae subclaviae stimulation of 2 and 20 Hz produced stepwise increases in CVR and in levels and overflows of the neurotransmitters (NPY and norepinephrine) in protocols 1A and B (Table 1). The venous concentrations of NPY (290.3 ± 77.3 pmol/liter) and the amounts of NPY overflow (7.38 ± 2.61 pmol/min per 100 g) were ~1,000-fold less than those of norepinephrine (148.3 ± 113.0 nmol/liter and 6.37 ± 4.29 nmol/min per 100 g) during 20-Hz stimulation under beta-blockade. In the presence of BIBP3226 (protocol 1C), however, nerve stimulation did not cause the significant increase of CVR, whereas the stimulation increased levels and overflows of the neurotransmitters. Nerve stimulation did not significantly change the subepicardial/subendocardial CVR ratio.

CVR correlated significantly with venous and arterial concentrations of NPY under alpha- and beta-blockade (protocol 1A) but not under beta-blockade (protocol 1B) (Table 2, Fig. 1). However, norepinephrine significantly correlated with CVR under intact alpha-adrenoceptor (under beta-blockade) but did not correlate under alpha- and beta-blockade. Under alpha- and beta-blockade, the values of the correlation coefficient (r) were highest for the venous NPY levels ($r = 0.850$) among the three indexes (arterial concentration, venous concentration and overflow). However, the determinant coefficient (r^2) was relatively low ($r^2 = 0.723$). In contrast, the venous norepinephrine level correlated with CVR with lower correlation and determinant coefficients ($r = 0.732$, $r^2 = 0.536$) under beta-blockade than those of the NPY-CVR correlation under alpha- and beta-blockade. The treatment of BIBP3226 abolished the correlation between NPY levels and CVR under alpha- and beta-blockade (protocol 1C, Fig. 1C). We also evaluated the correlations of CVR in the subepicardial and subendocardial regions with the indexes of the neurotransmitter. There was no obvious difference between the two regions; the r^2 values were 0.753 and 0.685 for venous concentration of NPY in the subepicardial and subendocardial regions under alpha- and beta-blockade, respectively, and 0.454 and 0.476 for venous norepinephrine under beta-blockade, respectively. The values of %CVR also correlated with the degree of increase in norepinephrine or NPY on stimulation, where the degree of increase was defined by the difference between the value during the stimulation and that at baseline. However, the r values were always less than those of the absolute values (data not shown). Logarithmic transformation did not improve the correlation coefficients in any indexes.

Role of the K_{ATP} channel in endogenous NPY stimulation of coronary vasoconstriction (protocol 2). Even in the presence of K_{ATP} channel modulators, ansae subclaviae stimulation also increased CVR and levels and overflows of the neurotransmitters in protocol 2 (Table 1). Glibenclamide treatment caused a significantly larger increase of %CVR in the subepicardial region during nerve stimulation than in the subendocardial region, whereas pinacidil treatment resulted the opposite effect at 20-Hz stimulation.

The mean value of CVR, including the data from the baseline, at 2-Hz and 20-Hz stimulations in the glibenclamide treatment group, was significantly higher than that in the pinacidil treatment group (2.38 ± 1.17 [21 points in seven dogs] vs. 0.87 ± 0.29 mm Hg/ml per min per 100 g [21 points in seven dogs], $p < 0.0001$ unpaired *t* test). However, between the two groups significant differences in mean venous NPY levels (271.1 ± 128.5 vs. 305.1 ± 171.8 pmol/liter) or mean venous norepinephrine levels (30.5 ± 42.4 vs. 28.9 ± 36.9 nmol/liter) were not observed (unpaired *t* test). Unlike protocol 1, there was no significant correlation between CVR and neurotransmitters in protocol 2. For example, the r values between CVR and venous NPY concentration with glibenclamide and pinacidil were 0.158 and 0.111, respectively (data not shown).

Reactivity of coronary vessels to exogenous NPY (protocol 3). Intracoronary administration of exogenous NPY increased CVR in a dose-dependent manner and revealed a more potent vasoconstrictive action than the same molar concentration of norepinephrine, both under alpha- and beta-blockade (protocol 3A, $p < 0.001$ ANCOVA) and under beta-blockade (protocol 3B, $p < 0.001$ ANCOVA) (Fig. 2). The concentration of NPY that increased CVR by 20% was ~100-fold lower than that of norepinephrine under beta-blockade. Intracoronary administration of exogenous norepinephrine caused only a negligible change under alpha- and beta-blockade (0.98 ± 0.18 mm Hg/ml per min per 100 g at baseline, $n = 5$) except for the maximal dose (0.3 μg/kg per min), but it caused a dose-dependent increase of CVR under beta-blockade (1.49 ± 0.52 mm Hg/ml per min per 100 g at baseline, $n = 10$) (ANOVA). The dose responsiveness of %CVR to norepinephrine was significantly suppressed by alpha-blockade ($p < 0.001$, ANCOVA) but that to NPY was not significantly suppressed ($p = 0.55$, ANCOVA). During administration, heart rate did not change significantly, nor did perfusion pressure, except for a slight increase of perfusion pressure at the maximal dose of NPY in protocol 3B (93.5 ± 18.6 to 100.0 ± 16.7 mm Hg, $n = 10$) and of norepinephrine in protocols 3A (84.4 ± 14.8 to

Table 1. Hemodynamic Variables During Ansae Subclaviae Stimulation in Protocols 1 and 2

	Protocol 1A	Protocol 1B	Protocol 1C	Protocol 2	
	Alpha- and Beta-Blockade (7 dogs)	Beta-Blockade (7 dogs)	Alpha- and Beta-Blockade Plus BIBP3226 (7 dogs)	Alpha- and Beta-Blockade Plus Glibenclamide (7 dogs)	Alpha- and Beta-Blockade Plus Pinacidil (7 dogs)
Heart rate (beats/min)					
Baseline	101.3 ± 34.1	105.7 ± 16.4	110.3 ± 11.7	101.4 ± 21.5	111.2 ± 21.0
2 Hz	108.5 ± 26.3	109.6 ± 16.1	111.1 ± 18.5	100.4 ± 20.4	115.5 ± 20.0
20 Hz	117.9 ± 23.6*†	118.7 ± 22.0*†	141.1 ± 24.5*†	101.9 ± 22.9	122.2 ± 24.9*
Mean aortic pressure (mm Hg)					
Baseline	70.1 ± 24.9	109.4 ± 19.1	77.0 ± 12.5	85.7 ± 14.2	61.6 ± 21.4
2 Hz	78.9 ± 30.0	113.6 ± 16.4	81.7 ± 8.1	99.9 ± 13.5*	63.4 ± 24.7
20 Hz	92.8 ± 32.7*†	122.9 ± 16.6*†	90.0 ± 14.5	109.1 ± 16.0*	71.3 ± 27.8*†
Coronary blood flow (ml/min per 100 g)					
Baseline	54.7 ± 18.2	61.3 ± 8.0	89.7 ± 49.5	51.5 ± 14.8	83.8 ± 26.9
2 Hz	59.8 ± 22.0	54.9 ± 8.7	69.2 ± 31.5	46.3 ± 22.5	76.6 ± 28.2
20 Hz	54.5 ± 20.0	53.5 ± 11.6	116.0 ± 29.1†	45.4 ± 19.0	76.3 ± 28.6
CVR (mm Hg/ml per min per 100 g)					
Baseline	1.35 ± 0.55	1.80 ± 0.30	1.08 ± 0.53	1.75 ± 0.47	0.78 ± 0.31
2 Hz	1.53 ± 1.04	2.10 ± 0.35	1.35 ± 0.44	2.58 ± 1.31	0.87 ± 0.29
20 Hz	1.96 ± 1.13*	2.40 ± 0.64*	0.81 ± 0.19†	2.82 ± 1.37*	0.96 ± 0.27*
CVR ratio (subepi/subendo)					
Baseline	1.45 ± 0.69	1.38 ± 0.31	1.33 ± 0.70	1.39 ± 0.19	1.21 ± 0.08
2 Hz	1.36 ± 0.52	1.28 ± 0.22	1.23 ± 0.39	1.73 ± 0.31	1.29 ± 0.20
20 Hz	1.31 ± 0.38	1.31 ± 0.15	1.33 ± 0.51	1.99 ± 0.56*	1.12 ± 0.09
Arterial NPY (pmol/liter)					
Baseline	161.1 ± 92.9	144.1 ± 59.9	302.7 ± 108.2	219.3 ± 73.1	285.3 ± 161.3
2 Hz	172.6 ± 98.7	137.4 ± 59.0	330.7 ± 127.8	208.6 ± 109.6	282.6 ± 146.2
20 Hz	203.6 ± 138.0	155.6 ± 58.3	377.1 ± 146.6	228.9 ± 102.0	298.9 ± 181.4
Venous NPY (pmol/liter)					
Baseline	159.7 ± 106.8	156.9 ± 66.3	221.6 ± 113.8	201.7 ± 80.6	258.1 ± 161.9
2 Hz	224.0 ± 134.7	169.6 ± 52.6	272.1 ± 134.0	224.7 ± 83.8	281.3 ± 160.1
20 Hz	369.6 ± 227.1*†	290.3 ± 77.3*†	492.4 ± 166.6*†	386.9 ± 133.4*†	375.9 ± 193.4*†
NPY overflow (pmol/min per 100 g)					
Baseline	−0.39 ± 4.28	0.74 ± 1.81	−6.15 ± 3.43	−1.08 ± 1.65	−2.46 ± 3.08
2 Hz	2.80 ± 4.17	1.86 ± 1.66	−3.97 ± 5.24	−0.42 ± 2.91	−0.14 ± 2.95
20 Hz	7.53 ± 4.11*	7.38 ± 2.61*†	14.30 ± 11.88*†	7.43 ± 7.93*†	7.01 ± 8.42*†
Arterial NE (nmol/liter)					
Baseline	4.5 ± 5.4	1.1 ± 0.5	2.4 ± 2.6	3.1 ± 2.3	3.9 ± 2.7
2 Hz	5.3 ± 5.2	1.7 ± 0.8	4.0 ± 2.2	4.8 ± 3.6	4.8 ± 2.4
20 Hz	12.0 ± 6.9*†	12.2 ± 5.6*†	18.5 ± 10.3*†	10.8 ± 3.8*†	17.3 ± 14.4*†
Venous NE (nmol/liter)					
Baseline	4.4 ± 9.3	0.9 ± 0.4	0.9 ± 0.6	2.5 ± 1.9	3.8 ± 2.1
2 Hz	23.4 ± 24.3	7.6 ± 4.1	28.9 ± 12.7	9.5 ± 2.0	11.1 ± 4.1
20 Hz	100.7 ± 58.8*†	148.3 ± 113.0*†	200.3 ± 134.7*†	79.4 ± 41.9*†	71.9 ± 35.6*†
NE overflow (nmol/min per 100 g)					
Baseline	−0.04 ± 0.29	−0.02 ± 0.03	−0.10 ± 0.14	−0.03 ± 0.04	0.02 ± 0.26
2 Hz	0.95 ± 0.99	0.31 ± 0.22	1.88 ± 1.40	0.19 ± 0.13	0.50 ± 0.39
20 Hz	4.74 ± 3.02*†	6.37 ± 4.29*†	19.61 ± 14.71*†	3.34 ± 2.51*†	3.78 ± 1.55*†

*$p < 0.05$ versus baseline value. †$p < 0.05$ versus value at 2 Hz (by analysis of variance). Data are expressed as mean value ± SD. See Methods for definitions of protocols 1 and 2. CVR = coronary vascular resistance; NE = norepinephrine; NPY = neuropeptide Y; subendo = subendocardium; subepi = subepicardium.

88.8 ± 17.9 mm Hg, n = 5) and B (105.1 ± 25.4 to 113.5 ± 24.2 mm Hg, n = 10) ($p < 0.05$, ANOVA).

Role of K_{ATP} channel in exogenous NPY stimulation of coronary vasoconstriction (protocol 4). As shown in Figure 3, treatment with the K_{ATP} channel antagonist glibenclamide significantly enhanced %CVR during NPY administration ($p < 0.001$, ANCOVA), whereas the agonists pinacidil or nicorandil produced a significant reduction ($p < 0.001$, ANCOVA) under beta-blockade (protocol 4A). There was no significant difference in the effects of pinacidil and nicorandil on NPY dose response (n = 3 and n = 3, respectively). The degree of suppression in vasoconstriction by nicorandil was directly proportional to the concentration of NPY and norepinephrine. NPY was more sensitive to the effect of nicorandil than norepinephrine ($p < 0.001$, ANCOVA); that is, 15% suppression in CVR was obtained at ~0.01 nmol/kg per min for

Table 2. Correlation Between Coronary Vascular Resistance and the Neurotransmitters (neuropeptide Y and norepinephrine) in Protocol 1

	Correlation (vs. CVR)			
	r Value	r^2 Value	p Value	Sy·x/ȳ
Alpha- and Beta-Blockade (protocol 1A [21 points in 7 dogs])				
Arterial NPY	0.680	0.462	0.0007	0.436
Venous NPY	0.850	0.723	0.0001	0.313
NPY overflow	0.285	0.081	NS	0.570
Arterial NE	−0.166	0.027	NS	0.587
Venous NE	−0.030	0.001	NS	0.594
NE overflow	0.090	0.008	NS	0.593
Beta-Blockade (protocol 1B [21 points in 7 dogs])				
Arterial NPY	−0.217	0.047	NS	0.238
Venous NPY	0.053	0.003	NS	0.243
NPY overflow	0.112	0.013	NS	0.242
Arterial NE	0.579	0.335	0.006	0.199
Venous NE	0.732	0.536	0.0002	0.166
NE overflow	0.674	0.454	0.0008	0.180
Alpha- and Beta-Blockade Plus BIBP3226 (protocol 1C [21 points in 7 dogs])				
Arterial NPY	0.268	0.072	NS	0.416
Venous NPY	−0.077	0.006	NS	0.431
NPY overflow	−0.282	0.080	NS	0.414
Arterial NE	−0.169	0.028	NS	0.426
Venous NE	−0.276	0.067	NS	0.415
NE overflow	−0.351	0.123	NS	0.405

NS = not significant (p value > 0.05); Sy·x/ȳ = residual error normalized to the mean; other abbreviations as in Table 1.

NPY and at an ~100 times higher dose (1.0 nmol/kg per min) for norepinephrine (protocol 4B, Fig. 4).

Discussion

New observations from this study. We have demonstrated that during sympathetic nerve stimulation the vasoconstrictive actions of NPY are masked by norepinephrine under intact alpha-adrenoceptor conditions and become manifest during alpha-blockade. The actions of both exogenous and endogenous NPY on the reactivity of coronary vessels were modulated by K_{ATP} channel activity.

Coronary vasoconstrictive action of endogenous NPY in vivo. We quantitatively evaluated the vasoconstrictive effect of both endogenous and exogenous NPY on coronary vessels in vivo. In the presence of alpha-blockade, the endogenous NPY level correlated significantly with CVR (r^2 = 0.723) during nerve stimulation (protocol 1A). It was NPY, not norepinephrine, that determined CVR at this condition, because 1) the norepinephrine level did not correlate with CVR whereas NPY did correlate in protocol 1A, 2) NPY Y_1-antagonist BIBP3226 blocked the nerve stimulation–induced coronary vasoconstriction and abolished the significant correlation between NPY levels and CVR in protocol 1C, 3) alpha-blockade by phenoxybenzamine attenuated the effect of phenylephrine by >30-fold (see Methods) and almost abolished the effect of exogenous norepinephrine in protocol 3A, and 4) exogenous NPY maintained a potent vasoconstrictive action despite the presence of alpha-blockade (protocol 3). Macho et al. (20) also reported that phentolamine did not modify the coronary vasoconstrictive effect of NPY.

Under intact alpha-adrenoceptor, however, endogenous NPY levels did not correlate with CVR, whereas norepinephrine levels showed good correlation (protocol 1B). This difference may be due to the low level of release or the low potency of NPY compared with norepinephrine during nerve stimulation. The former possibility could be explained by the results obtained during ansae subclaviae stimulation where NPY overflow was ~1,000-fold lower than norepinephrine overflow in protocol 1, assuming the overflow to be an index of the level of release. NPY occurred only in large, dense core vesicles, in which the molar ratio of NPY to norepinephrine was ~1:200 (4,21,22). To investigate the latter possibility, we examined the vasoconstrictive effect of NPY and norepinephrine independently in protocol 3, where NPY had an ~100-fold greater effect than norepinephrine. This value was comparable to the results obtained in the isolated perfused dog heart (20).

The rough correlation between norepinephrine levels and CVR (r^2 = 0.536) under intact alpha-adrenoceptor indicated that norepinephrine can account for only 54% of the variation in CVR during the stimulation, which is less than that of NPY under alpha-blockade. NPY probably contributes to some of the residual 46% of variation. Thus, the vasoconstrictive actions of endogenous NPY may be masked by norepinephrine under intact alpha-adrenoceptor and is manifest only under alpha-blockade. Under intact alpha-adrenoceptor, Otani et al. (13) deduced that the quantity of released NPY during sympathetic nerve stimulation was insufficient to elicit a coronary vasoconstriction based on their indirect observations that 1) after cessation of sympathetic nerve stimulation, CVR returned rapidly to its control value, 2) coronary constriction by exogenous NPY was sustained for 48 min, 3) intense antecedent sympathetic stimulation did not alter the coronary vascular responses to subsequent norepinephrine infusions, and 4) the modulation of the vagal effect on heart rate induced by sympathetic nerve stimulation persisted for ~1 h. In the present study, however, we directly demonstrated NPY-induced coronary vasoconstriction by showing a quantitative correlation between NPY level and CVR under alpha-blockade without NPY antagonist (protocol 1A) and lack of correlation with NPY antagonist (protocol 1C). This vasoconstrictive action was masked by the effects of norepinephrine under intact alpha-adrenoceptor (protocol 1B).

CVR had correlated better with the venous concentration of NPY and norepinephrine than with the overflow of these transmitters. This finding suggested that the regulation of NPY and norepinephrine levels coupled to adrenergic receptors relies on the local venous concentrations rather than on the amount of overflow. The local concentrations of NPY and norepinephrine are determined by the quantity of release, the rate of degradation, reuptake and washout. Our present findings suggest that the rate of washout, or the flow rate in the

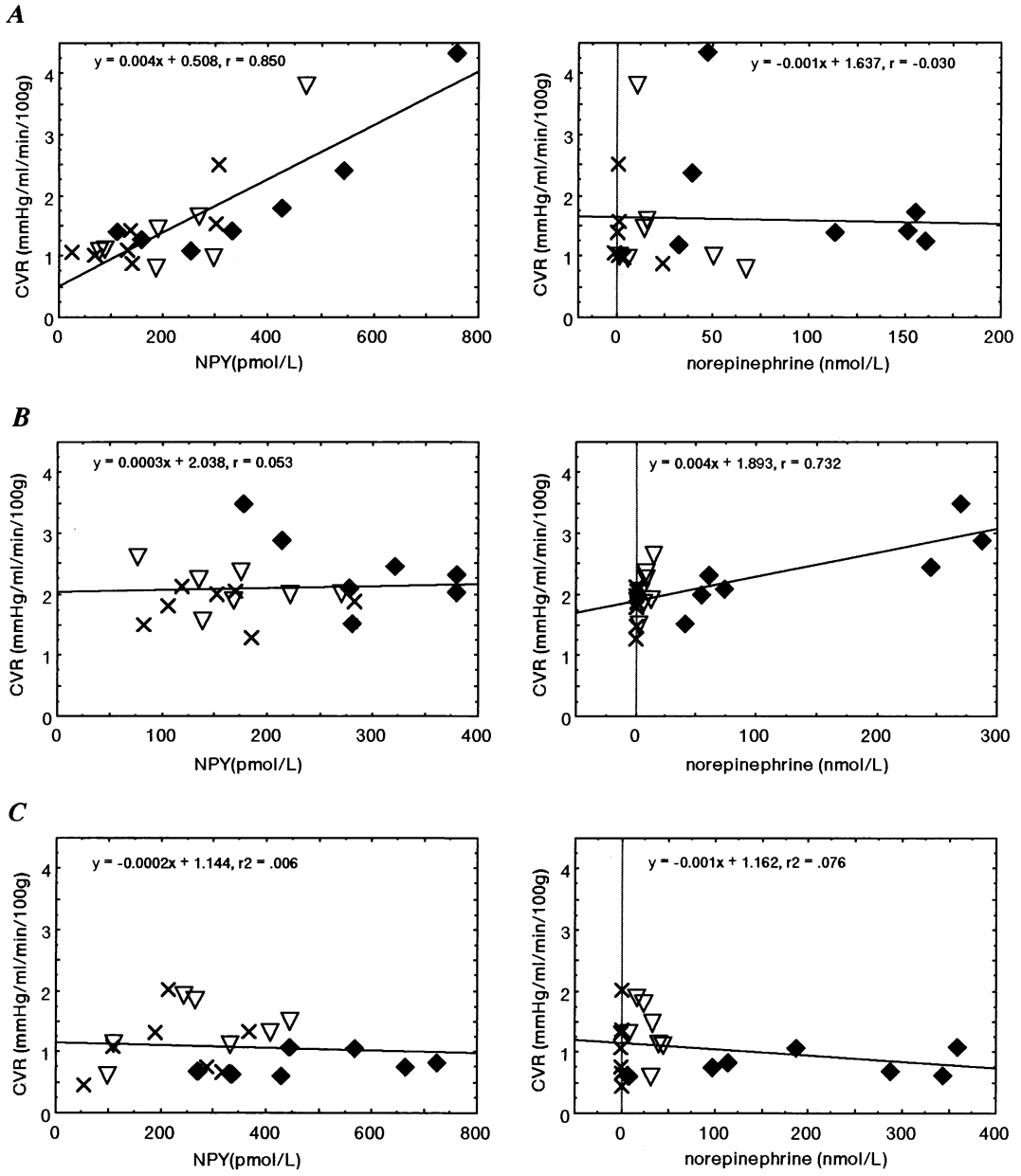

Figure 1. Correlation of venous levels of neuropeptide Y (NPY, **left panels**) and norepinephrine (**right panels**) with coronary vascular resistance (CVR). **A,** Alpha- and beta-blockade in protocol 1A (seven dogs). **B,** Beta-blockade in protocol 1B (seven dogs). **C,** Alpha- and beta-blockade plus BIBP3226 in protocol 1C (seven dogs). **Crosses** = baseline values; **triangles** = 2-Hz stimulation; **diamonds** = 20-Hz stimulation. L = liter.

tissue, modifies the local concentrations of NPY and norepinephrine and thereby CVR. Released NPY was oxidized or degraded into fragments (23). Piedimonte et al. (24) reported that neutral endopeptidase inhibition, which probably reduced degradation of bradykinin, potentiated sensory nerve–induced coronary vasodilation. However, nothing is known about modification of NPY-induced coronary vasoconstriction by a specific peptidase. Reuptake of NPY by nerve terminals is also unlikely.

The role of K_{ATP} channel in the modulation of CVR. The relatively rough correlation between NPY levels and CVR ($r^2 = 0.723$) indicated that NPY can account for only 72% of the variation in CVR during sympathetic nerve stimulation. Other mechanisms may play additional modulatory roles for CVR. The present results in protocols 2 and 4 indicate that the K_{ATP} channel modulates CVR by changing the reactivity of coronary vessels to NPY. A K_{ATP} channel antagonist enhanced CVR and agonists attenuated CVR during administration of exogenous NPY (protocol 4), and these substances modified CVR without changing overflow or local levels of NPY or

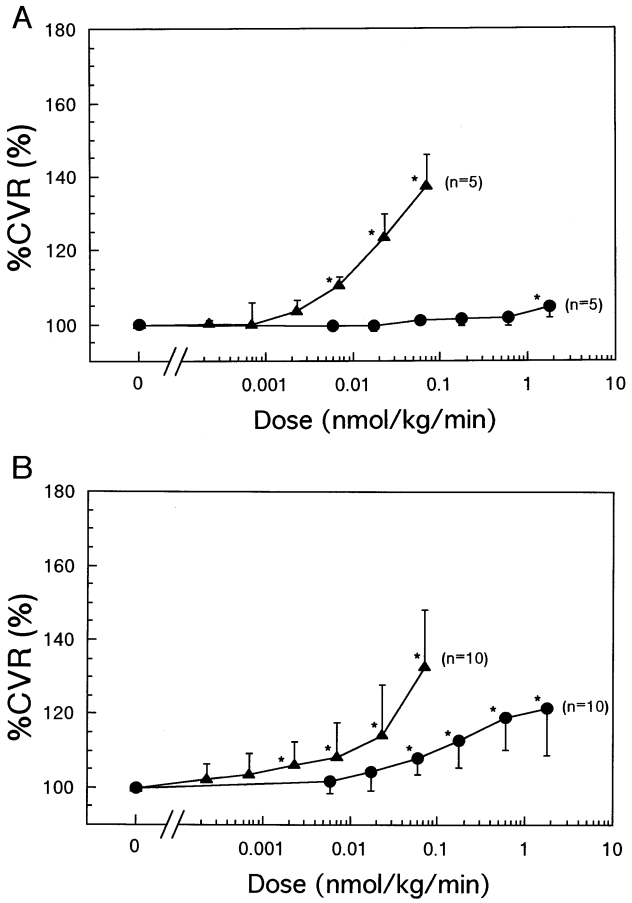

Figure 2. Dose-response curves of percent changes in coronary vascular resistance (%CVR) with intracoronary administration of exogenous neuropeptide Y and norepinephrine in protocol 3. Neuropeptide Y **(triangles)** and norepinephrine **(circles)** were administered to the coronary artery under alpha- and beta-blockade **(A)** and under beta-blockade **(B). Symbols** and **bars** represent mean values and SD, respectively, for the number of dogs indicated in parentheses. ANCOVA was applied to examine the differences in the dose-response curves ($p < 0.001$, neuropeptide Y vs. norepinephrine). *$p < 0.05$ versus baseline value (ANOVA).

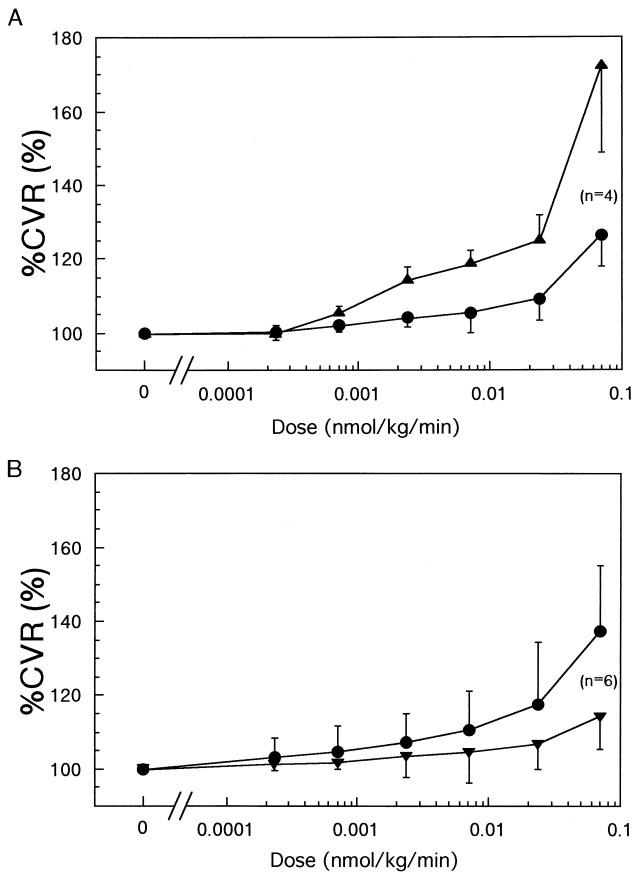

Figure 3. Effect of adenosine triphosphate–sensitive potassium (K_{ATP}) channel acting substances on the dose responsiveness of percent changes in coronary vascular resistance (%CVR) induced by intracoronary administration of neuropeptide Y under beta-blockade in protocol 4A. **A,** Effect of K_{ATP} channel antagonist glibenclamide **(triangles)** in four dogs. **B,** Effect of K_{ATP} channel agonist **(triangles)** pinacidil (three dogs) or nicorandil (three dogs). Control experiments **(circles)** were done in the same dogs (data from protocol 3B). **Symbols** and **bars** represent mean value and SD, respectively. ANCOVA was applied to examine the differences in the dose-response curves: **A,** $p < 0.001$, glibenclamide versus control; **B,** $p < 0.001$, K_{ATP} versus control.

norepinephrine during ansae subclaviae stimulation (protocols 1 and 2). It has been reported that substances that act on K_{ATP} channels may modify vessel reactivity to norepinephrine (16) and to adenosine (14). In this study, we demonstrated a new observation that K_{ATP} channel activity modifies the effects of NPY (and norepinephrine) on vascular tone. As shown in Figure 4 (protocol 4B), the effects of NPY were more markedly (~100-fold) suppressed by K_{ATP} channel agonists than were those of norepinephrine, indicating that the proportion of K_{ATP} channel dependency in the vasoconstrictive action of NPY is greater than that of norepinephrine. This observation suggests that NPY-induced vasoconstriction would become more marked than that of norepinephrine under the K_{ATP} channel blockade of coronary vessels. Modification of CVR by the K_{ATP} channel was more marked in the subepicardial than in the subendocardial region during ansae subclaviae stimulation (Table 1). These results indicate that there is transmural heterogeneity in the mechanism regulating CVR by way of K_{ATP} channels, as discussed previously (16).

Possible modulation of coronary vasoconstriction by other transmitters during nerve stimulation. As well as K channels, some other neurotransmitters should be considered as additional modulatory factors for CVR during electrical nerve stimulation. ATP is coreleased from the sympathetic nerve (4,25) and is immediately degraded into adenosine. Adenosine is also produced in myocardium by ischemia (26). Calcitonin gene-related peptide is released from the sensory nerve by antidromic nerve stimulation (27). ATP caused vasoconstriction (28), whereas adenosine (29,30) and calcitonin gene–related peptide (31,32) caused vasodilation. Thus, these three substances possibly modulate CVR during electrical nerve stimulation. However, we have already reported that electrical stimulation of the ventrolateral cardiac nerve increased

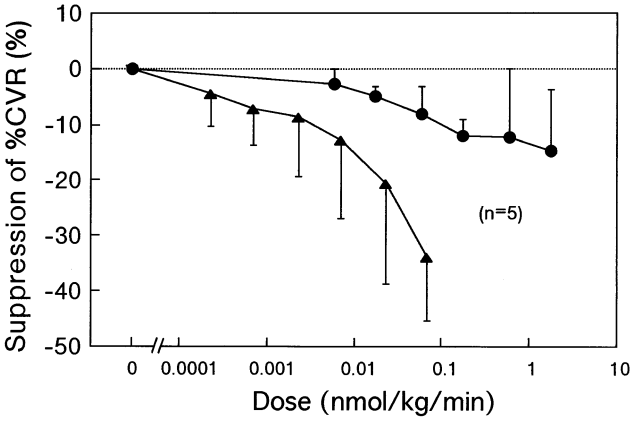

Figure 4. Effect of the adenosine triphosphate–sensitive potassium channel agonist nicorandil on the dose-responsiveness of percent changes in coronary vascular resistance (%CVR) induced by intracoronary administration of neuropeptide Y and norepinephrine in protocol 4B. Neuropeptide Y (**triangles**) and norepinephrine (**circles**) were administered to the coronary artery under beta-blockade in five dogs. The degree of suppression was estimated by the difference in %CVR between the conditions with and without nicorandil. **Symbols** and **bars** represent mean value and SD, respectively. ANCOVA was applied to examine the differences in the dose-response curves ($p < 0.001$, neuropeptide vs. norepinephrine).

CVR in a frequency-dependent manner, where neither 8-phenyltheophylline (adenosine antagonist) nor calcitonin gene–related peptide 8-37 (calcitonin gene–related peptide antagonist) modified CVR change compared with the control series (16). In the preliminary experiments, we confirmed that intracoronary administration of alpha, beta-methylene ATP (ATP antagonist [1.0 μg/kg per min]) had no effect on CVR change during the ventrolateral cardiac nerve stimulation. Other factors such as prostacyclin, endothelium-derived relaxing factor, endothelium-derived hyperpolarizing factor, endothelin and others might be involved in the regulating mechanisms. Sympathetic nerve stimulation augmented the release of prostacyclin but did not affect the release of endothelium-derived relaxing factor or endothelin in rabbit Langendorff hearts (33). Inhibition of prostaglandin biosynthesis by ketoprofen or aspirin caused a paradoxic increase in CVR by cold pressor test in humans (34). Inhibition of nitric oxide production by N^{ω}-nitro-L-arginine–enhanced nerve stimulation induced norepinephrine release in rat Langendorff hearts (35) but had no effect on CVR change during the ventrolateral cardiac nerve stimulation in dogs in vivo (16). Little is known concerning modulation of sympathetic coronary vasoconstriction by endothelium-derived hyperpolarizing factor, endothelin or other substances.

Consideration of the experimental model. To avoid metabolic regulatory mechanisms, we stimulated sympathetic nerves locally and used beta-blockade. In protocol 3, CVR increased with negligible changes in heart rate and aortic pressure, exhibiting almost complete independence of metabolic regulation. In protocol 1, the ansae subclaviae stimulation caused systemic hemodynamic changes even under beta-blockade, and metabolic vasodilation consequently occurred. However, beta-blockade at least minimized the increases in heart rate (11%) and aortic pressure (13%, Table 1) exhibited during sympathetic stimulation.

Alpha-blockade with phenoxybenzamine masked the effects of the exogenous norepinephrine in protocol 3A and competitively suppressed the effect of phenylephrine on vasoconstriction as described in Methods (the threshold of phenylephrine increased >30-fold), but it did not completely suppress the augmentation of aortic pressure during ansae subclaviae stimulation in protocol 1A. In other words, a few functioning alpha-adrenoceptors are likely to have been present around the synapse area. However, a lack of correlation between norepinephrine and CVR under alpha-blockade indicates that the blockade was sufficient for the present conclusions related to NPY. NPY blockade with BIBP3226 enhanced the increase in heart rate and neurotransmitter overflows at 20 Hz. These changes were probably due to suppression of presynaptic inhibition of transmitter release by way of the NPY receptor.

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