α_2-Adrenoceptors control the release of noradrenaline but not neuropeptide Y from perivascular nerve terminals

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Abstract

Neuropeptide Y (NPY) and noradrenaline (NA) are co-transmitters at many sympathetic synapses, but it is not yet clear if their release is independently regulated. To address this question, we quantified the electrically evoked release of these co-transmitters from perivascular nerve terminals to the mesenteric circulation in control and drug-treated rats. 6-Hydroxydopamine reduced the tissue content and the electrically evoked release of ir-NPY and NA as well as the rise in perfusion pressure. A 0.001 mg/kg reserpine reduced the content of ir-NPY and NA, but did not modify their release nor altered the rise in perfusion pressure elicited by the electrical stimuli. However, 0.1 mg/kg reserpine reduced both the content and release of NA but decreased only the content but not the release of ir-NPY; the rise in perfusion pressure was halved. Clonidine did not affect the release of ir-NPY while it lowered the outflow of NA, not altering the rise in perfusion pressure elicited by the electrical stimuli. Yohimbine, did not modify the release of ir-NPY but increased the NA outflow, it antagonized the clonidine effect. Therefore, presynaptic α_2-adrenoceptors modulate the release of NA but not NPY, implying separate regulatory mechanisms.

Index terms: Neuropeptide Y (NPY) release; Noradrenaline (NA) release; Sympathetic co-transmitter release; Reserpine; 6-Hydroxydopamine; α_2-Presynaptic receptors; Presynaptic regulation; α_2-Autoreceptors

1. Introduction

The sympathetic nervous system regulates the vascular tone by releasing neuropeptide Y (NPY), noradrenaline (NA), and ATP as co-transmitters; these signals act post-junctionally in a coordinated fashion resulting in a fine regulation of the vascular smooth muscle [30]. In addition, NPY, NA, and ATP or adenosine may also act at the nerve terminal through presynaptic receptors, regulating the release of transmitters. The α_2-adrenoceptor was the first of these receptors to be characterized both functionally and pharmacologically and was found to inhibit the release of NA [23,24]. The concept of presynaptic regulatory receptors and of autoreceptors was then extended to central and peripheral transmitters such as serotonin, acetylcholine, GABA, adenosine, the opioid peptides, angiotensin II, bradykinin, and NPY, among others [17,20]. Some presynaptic receptors decrease transmitter release, while others increase the output of transmitters. This functional difference apparently depends on the coupling of these receptors to G-proteins and on their intracellular transduction pathways. However, the role and functional properties of presynaptic receptors remain obscure.

Abundant evidence supports the contention that NA is found in both the small and the larger dense cored vesicles, while NPY appears to be exclusively stored in the large vesicles [6,11,18]. Early studies addressing the correlation between the frequency of nerve terminal stimulation and co-transmitter release revealed that the small vesicles require lower frequencies for their release compared to those storing NPY [18]. Later studies refuted this notion, finding that the ratio of NA/NPY released was not dependent on the frequency of nerve terminal stimulation [7]. Consequently, they raised the idea that only the large vesicles are released from the peripheral sympathetic nerve terminals, and that this release required the fusion of small vesicles with immature large vesicles. Regarding the release of ATP, the third integrant of sympathetic co-transmission, few studies have been reported. A different time course of co-release was found in a study analyzing the output of NA and ATP [26], suggesting co-transmitter storage in two types of vesicles, one containing more ATP than NA and the other containing more NA than ATP.

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Another approach used to characterize sympathetic co-transmitter release and investigate putative storage mechanisms is to study the modulator role of presynaptic receptors in transmitter release. Upon electrical nerve stimulation of peripheral autonomic synapses; α-adrenoceptor blockade enhances the output of NA and NPY to the blood stream [19]. Recently, Brock et al. [3] found that the release of both NA and ATP is increased by β_2-adrenoceptor stimulation in the rat-tail artery. Other studies show that distinct mechanisms control the release of NA and ATP. In rat mesenteric arteries, α_2-adrenoceptor activation differentially modulates the release of NA and ATP [10]; likewise, in the rabbit vas deferens, angiotensin II increases the release of NA while reducing the purinergic component [28]. In further support of the latter study, in the guinea pig vas deferens, β-adrenoceptor stimulation increased the NA released but decreased ATP output [15]. These results show differential modulation of co-transmitter release and imply a separate vesicular storage for each co-transmitter. However, the latter studies did not determine the release of NPY.

Based on the observation that only the large dense-core vesicles store NPY [6], we hypothesized that, depending on the co-transmitter content of each vesicle, the release of NPY and NA is differentially regulated. To explore the mechanism(s) that regulate the electrically evoked release of NPY and NA, we used 6-hydroxydopamine and reserpine to distinguish between NPY and NA storage, and to clarify whether their release is altered to a similar extent by these drugs. We also investigated whether α_2-adrenoceptor activation with clonidine, which is known to inhibit the release of NA, would reduce in a parallel fashion the electrically evoked release of NPY and NA. As an experimental model we used the sympathetic perivascular nerve terminals surrounding the rat mesenteric artery, which we previously characterized as a reliable model to measure the release of ir-NPY and NA evoked by electrical depolarization [8]. This preparation is also suited to monitor the functional response following electrically evoked co-transmitter release. The present results demonstrate that a 48-h pre-treatment with 0.1 mg/kg reserpine considerably reduces the nerve terminal content of both NPY and NA but abolishes only the electrically evoked release of NA without altering the release of ir-NPY. Furthermore, clonidine inhibited only the electrically evoked release of NA without modifying the release of ir-NPY. Altogether these results are consistent with the contention that the release of ir-NPY and NA is regulated by distinct presynaptic mechanisms.

2. Materials and methods

2.1. Immuno-histochemical assays in the isolated arterial and vein tree

Sprague–Dawley rats were anesthetized with 40 mg/kg pentobarbitone i.p. The mesenteric arcade was removed by blunt dissection and placed in Krebs–Ringer solution. A large piece of fat overlaying the superior mesenteric artery was eliminated. Adipose tissue surrounding the arteries, arterioles, and large, branching veins was carefully detached by stripping with forceps [5], allowing the separation of the arterial tree from the vein tree. Flat whole mount preparations of the mesenteric vessels were carefully prepared. Defatted preparations were fixed in paraformaldehyde 4% plus picric acid 1% and placed in phosphate buffer. Tissues were incubated for 6 h with specific NPY or DβH antibodies (1/500 dilution). The second antibody (1/100 dilution), coupled to fluorescein, was incubated for an additional 3 h in the dark. Tissues were examined with a fluorescence microscope to reveal the presence of NPY and DβH immunoreactivity. Control tissues were prepared by either omitting primary or secondary antibodies from the incubation solutions. Parallel histochemical reactions for non-specific and specific cholinesterase were performed according to Karnovsky and Roots [16], to identify cholinergic nerve fibers surrounding the mesenteric artery, veins, and their corresponding ramifications. In brief, the mesenteries were fixed in paraformaldehyde 4% and glutaraldehyde 1%, put in phosphate buffer during 6 h, and incubated next for 3 h with acetylthiocholine iodide plus sodium citrate 100 mM, cupric sulfate 30 mM, and potassium ferrocyanide 5 mM. The brown color reaction was observed with light microscopy. Acetylcholinesterase specific staining was performed incubating the tissues for 3 h with 1 μM *iso*-OMPA, a butyrylcholinesterase inhibitor. To visualize butyrylcholinesterase, tissues were incubated for 3 h with 1 μM BW 284c51, a specific inhibitor of acetylcholinesterase.

2.2. Perfusion of the rat arterial mesenteric bed; collection of the luminally accessible NPY and NA released by electrical stimulation

Rat mesenteries were isolated and perfused as detailed by Donoso et al. [8]. The superior mesenteric artery was isolated and cannulated to initiate perfusion with oxygenated Tyrode buffer (37 °C) at a flow of 1 ml/min; perfusion pressure was monitored continually using a pressure transducer connected to a multichannel Grass polygraph. After a 30-min equilibration period, the perivascular nerve terminals surrounding the rat superior mesenteric artery were electrically depolarized (20 Hz, 1-ms trains of 60 V, for 1 min) by means of platinum electrodes connected to a Grass S44 stimulator. The effluent from the mesenteries was collected to determine the amount of ir-NPY and NA in the perfusion buffer before, during, and after the electrically evoked nerve stimulation. To quantify the luminally accessible NPY and NA, perfusates were collected every 4 min for ir-NPY or 2 min for NA determinations. For the detection of the ir-NPY, mesenteries were perfused with Tyrode buffer added with aprotinin (0.025 TIU/ml).

2.3. Extraction and chromatographic identification of NPY

To determine the content of NPY, isolated mesenteries were weighed and homogenized in 9 ml of 2 M acetic acid with 4% EDTA, as detailed by Donoso et al. [8]. NPY was extracted from intact mesenteries, and quantified using an established RIA [8], directed towards the NPY carboxy terminus, as characterized by the use of selective peptide fragments. Tissue NPY content and the luminally accessible NPY released by perimesenteric artery nerve terminal stimulation is expressed as ir-NPY.

Synthetic human or rat NPY standards (hNPY), hNPY fragments, porcine NPY fragments, and samples of mesenteric perfusates were separated by HPLC. A Hibar RP-18 column was equilibrated with 80% of mobile phase A (0.1% TFA) and 20% of mobile phase B (80% acetonitrile/0.1% TFA). The samples were eluted with the following gradient program: 0–5 min, 80% A–20% B; 5–10 min, 60% A–40% B; 10–40 min, 53% A–47% B; 40–60 min, 100% B. Before use, the mobile phases were degassed. A constant flow was maintained at 1 ml/min. Each ml of eluate was collected separately, dried in a speed vacuum, and stored at $-20\,^{\circ}$C. The samples were reconstituted separately in RIA buffer and quantified in duplicate by the RIA procedure. The results are expressed as the percentage ir-NPY *per* fraction with respect to the total ir-NPY of the assay.

2.4. Extraction and chromatographic identification of noradrenaline (NA)

After wet tissue weight, the samples were homogenized in 2 ml perchloric acid 0.2N and centrifuged at 2500 rpm for 20 min at 5 °C. The pellet was discarded and the supernatant was assayed to quantify NA by HPLC with electrochemical detection as described by Donoso et al [8]. The same procedure was used to quantify the amount of NA released to the perfusate.

2.5. NPY and NA content in defatted tissues

To assess the tissue content of NPY and NA in defatted mesenteries, the same procedure outlined above was used to prepare the tissue samples for the quantification of ir-NPY and NA.

2.6. Sympathetic origin of the co-transmitters

To confirm that NPY and NA are stored in sympathetic nerve terminals, rats were pre-treated with either 6-hydroxydopamine or reserpine. Mesenteries were prepared for perfusion as detailed above. The tissue content and the luminally accessible ir-NPY and NA released by electrical stimulation were determined.

2.6.1. 6-Hydroxydopamine

Under ether anesthesia, rats were injected 48 h prior to the experiment with 100 mg/kg of the neurotoxin dissolved in saline plus 1% ascorbic. The abdominal cavity was opened; a small incision allowed localization of the main mesenteric artery. Following drug injection, the abdominal cavity was sutured. Parallel rats were injected with the same volume of 1% ascorbic acid saline.

2.6.2. Reserpine

Groups of 3–8 rats were injected i.p. with 0.001, 0.01, 0.1, 1, and 2 mg/kg reserpine 48 h before ir-NPY and NA determinations.

2.7. Presynaptic α_2-adrenoceptor activation

To assess the influence of presynaptic α_2-adrenoceptors on the modulation of the NPY and NA released by electrical nerve depolarization, mesenteries were perfused for 30 min with clonidine prior to electrical stimulation of the perivascular nerve terminals with 20-Hz trains for 1 min. Perfusates were collected as detailed above to quantify the luminally accessible ir-NPY and NA released.

Mesenteries were perfused with 0.3, 1, 10, and 100 nM clonidine ($n = 4$–6 rats per group). To ascertain whether the effect of clonidine on co-transmitter release was dependent on α_2-adrenoceptor activation, yohimbine was used to antagonize these receptors. This protocol was carried perfusing simultaneously 10 nM clonidine plus 1 μM yohimbine ($n = 6$ for ir-NPY determinations) or 1 nM clonidine plus 1 μM yohimbine ($n = 4$ for NA determinations). In a separate group of rats, the effect of 1 μM yohimbine alone on ir-NPY and NA release was evaluated ($n = 4$).

2.8. Animals and drug sources

Experiments were conducted in accordance with the American NIH guidelines for experimental animal use as detailed in the NIH Guide for the Care and Use of Laboratory Animals; the University and the Faculty Animal Experimental Committees approved our animal protocols. We used male Sprague–Dawley rats (250–300 g) bred in the Faculty of Animal Reproduction Laboratories. Human NPY, NPY fragments, human–rat NPY antiserum, and normal rabbit serum were obtained from Peninsula Labs (Belmont, CA). Noradrenaline hydrochloride (NA), reserpine, clonidine hydrochloride, yohimbine hydrochloride, 6-hydroxydopamine, acetylthiocholine iodide, sodium citrate, cupric sulfate, potassium ferrocyanide, 1,5-bis(4-allyldimethyl-ammoniumphenyl) pentan-3-one dibrimide (BW 284c51), tetraisopropyl pyrophosphoramide (*iso*-OMPA), 3,4-dihydroxybenzylamine and aprotinin were purchased from Sigma. Alumina and buffer Krebs–Ringer reagents were purchased from Merck, Chile.

2.9. Expression of results and statistical analysis

Results are expressed as the mean value ± S.E.M. The Student's *t*-test was used to compare the effect of drug treatments to its control group. When appropriate, Dunnett's tables for multiple comparisons with a single control group were used. ANOVA was used to ascertain the significance of the outflow time course studies of co-transmitters released. Significance was set at a probability less than 0.05 (P-value < 0.05).

3. Results

3.1. Immunofluorescence of sympathetic nerve terminals and content of NA and NPY

Dense networks of NPY and DβH immunoreactive nerve terminals were observed around the arteries and veins of the rat mesentery in flat mount preparations. The density and pattern of the enzyme immunoreactivity is similar for both markers in arteries (Fig. 1) and veins but the intensity of the immunofluorescence is lower in veins (results not shown). Treatment with 6-hydroxydopamine abolished NPY and DβH immunoreactivities (Fig. 1), demonstrating the sympathetic origin of these nerve endings. Positive cholinesterase reaction was observed surrounding the mesenteric arterioles (Fig. 1) and venules (results not shown), suggesting the presence of cholinergic nerve terminals. Acetylcholinesterase fibers were identified since the staining persisted in the presence of *iso*-OMPA, a selective inhibitor of butyryl cholinesterase. In contrast, the histochemical reaction was lost when the mesenteries were incubated simultaneously with *iso*-OMPA plus BW 284c51, a selective acetylcholinesterase inhibitor. Acetylcholinesterase-containing nerve terminals are preserved following 6-hydroxydopamine treatment (Fig. 1), an indication that the toxin acts selectively on sympathetic nerve endings.

The ir-NPY content was similar in the arterial and venular defatted vascular preparations when expressed as pmol/g tissue (Table 1). However, in accordance with the immunofluorescence studies, when the ir-NPY tissue content is expressed independently of the tissue weight, the arteries have consistently more ir-NPY than the veins (8.4 ± 2.4 pmol, $n = 4$ versus 3.4 ± 1.3 pmol, $n = 6$, $P < 0.042$). In contrast, the content of NA expressed as pmol/g of tissue, was twice as large in the arterial tree as in the veins ($n = 4$, $P < 0.03$, Table 1). However, when the results are expressed independent of the tissue weight, the values were 265 ± 44 ($n = 4$) versus 60 ± 18 pmol ($n = 4$, $P < 0.0025$), indicating an even larger difference in the co-transmitter content between arteries and veins.

3.2. Release of co-transmitters

We next assessed the luminal release of the sympathetic co-transmitters elicited by electrical stimulation. A 20-Hz train of electrical pulses caused a sharp rise in perfusion pressure associated to an increase in the luminal outflow of ir-NPY and NA. The maximal rise in pressure reached 87.9± 8 mmHg ($n = 19$); not always the rise was maintained, it faded in 4/19 cases. Electrical stimulation of the perivascular nerve terminals evoked a significant release of luminally accessible ir-NPY ($F(5, 35) = 6.43$, $P < 0.0005$) and NA ($F(5, 35) = 4, 13$, $P < 0.007$). The time course of the luminally accessible increase in co-transmitter released indicates that NA recovers basal values within 6–8 min while NPY takes longer (Fig. 2). The HPLC chromatographic analysis

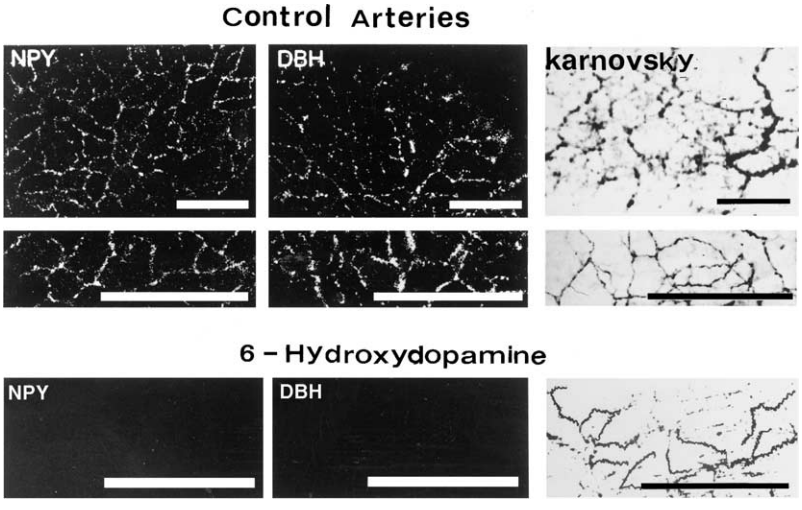

Fig. 1. NPY and dopamine β-hydroxylase immunoreactive and acetylcholinesterase histochemistry in mesenteric perivascular nerve terminals surrounding small arteries. Left and middle panels show NPY and dopamine β-hydroxylase (DBH) immunoreactivities. The right panel shows the Karnovsky reaction in the presence of *iso*-OMPA, to selectively identify acetylcholinesterase in control and in rats pre-treated with 6-hydroxydopamine 48 h previously. Upper panel: images from control, saline-treated rats. Lower panel: 6-hydroxydopamine treated animals. In both cases, the immunoreactive images are from a same mesentery, while the Karnovsky reaction was conducted in a separate preparation. Calibration bars indicate 500 μm.

Table 1
ir-NPY and NA content in the rat mesenteric bed

	ir-NPY (pmol/g tissue) ($x \pm$ S.E.M. (n))	NA (pmol/g tissue) ($x \pm$ S.E.M. (n))	Ratio NA/ir-NPY
Whole mesentery	28 ± 2.8 (15)	1296 ± 99 ($n = 8$)	46
Defatted tissues			
Arterial tree	133 ± 37 (4)	9900 ± 1600 (4)	74
Vein tree	105 ± 33 (5)	5300 ± 1200 (4)	50

of the perfusate confirmed the identity of the ir-NPY released by electrical nerve terminal depolarization (Fig. 3A and B). The retention time of the immunoreactive material eluted coincided with the retention time of a synthetic human NPY sample (Table 2). The specificity of the Peninsula NPY antibody for several peptide fragments is detailed in Table 2.

3.3. *Influence of 6-hydroxydopamine and reserpine on co-transmitter content and release*

3.3.1. *Reduction in the mesentery ir-NPY and NA content*

The mesentery content of ir-NPY and NA in non-treated rats was 28 ± 2.8 ($n = 15$) and 1296 ± 99 ($n = 8$ pmol/g tissue), respectively (Table 1). The ratio of NA/ir-NPY was 46:1. Consistent with the severe reduction in the ir-NPY and NA content observed histochemically, at least a 90% reduction in the content of these co-transmitters was measured after 6-hydroxydopamine treatment (3.9 ± 1.1 ($n = 7$) and 96.8 ± 45.4 ($n = 7$) pmol/g of tissue, respectively). Reserpine potently depleted the ir-NPY and NA content. A dose of 0.001 mg/kg reserpine reduced the content of ir-NPY and NA to 18.6 ± 2.8 ($n = 4$) and 712 ± 13 ($n = 3$) pmol/g of tissue, respectively, values which represent a 39 and 44% reduction, respectively. Larger doses of reserpine consistently induced a more substantial depletion of these co-transmitters (Fig. 4A); maximal reduction was ≈80–90%.

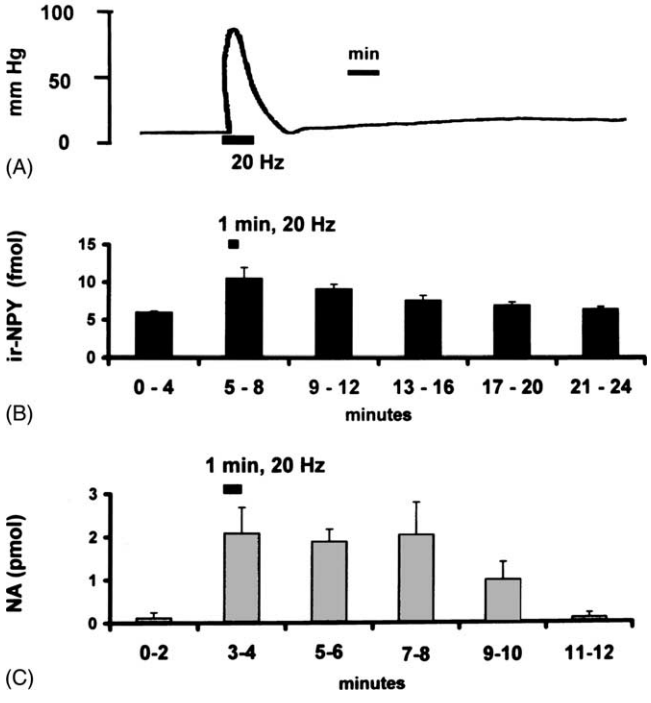

Fig. 2. Time course of the outflow of ir-NPY and NA evoked by electrical stimulation. (A) Polygraphic tracing shows the rise in perfusion pressure evoked by electrical stimulation of the mesenteric artery perivascular nerve terminals (20 Hz, 60 V, 0.5 ms) during 1 min. (B) Outflow of the electrically evoked luminally accessible ir-NPY ($n = 6$). ANOVA indicates a significant increase of ir-NPY outflow elicited by the electrical depolarization of the nerve terminals $F(5, 35) = 6.43$, $P < 0.0005$. (C) Outflow of NA in a paired series of mesenteries ($n = 6$); ANOVA showed a significant increase in the NA released $F(5, 35) = 4.13$, $P < 0.007$. Columns indicate mean values, bars the S.E.M.

3.3.2. *Reduction of ir-NPY and NA release*

6-Hydroxydopamine abolished the electrically evoked luminally accessible outflow of and ir-NPY ($n = 3$) and NA ($n = 5$). Consequently, a barely detectable rise in the perfusion pressure was observed in the chemically denervated mesenteries as compared to control preparations (4.4 ± 1.9 ($n = 8$) versus 87.9 ± 8 mmHg ($n = 19$); $P < 0.001$, respectively). Reserpine reduced the electrically evoked release of luminally accessible ir-NPY and NA (Fig. 4B); although 0.001 mg/kg reserpine reduced 40% the ir-NPY and NA content (Fig. 4A), this dose did not affect the release of the electrically evoked co-transmitters. Pre-treatment with 0.1 mg/kg reserpine was necessary to abolish the NA release; however, this dose did not modify the release of ir-NPY. A dose of 1 mg/kg reserpine was required to reduce the release of ir-NPY (Fig. 4B). The rise in perfusion pressure evoked by perivascular nerve stimulation was significantly lowered

Table 2
Characterization of the NPY antiserum specificity utilized in the RIA assay to quantify NPY and determine the HPLC retention time for NPY and its fragments

Peptide	Cross-reactivity (%)	Retention time (min)
NPY (human, rat)	100	39.5
NPY 2–36	100	35.8
NPY 11–36 (porcine)	100	42.3
NPY 13–36 (porcine)	100	36.5
NPY 16–36 (porcine)	100	37.1
NPY 18–36	100	22.7
NPY 20–36	100	24.1
NPY 25–36	40	15.1

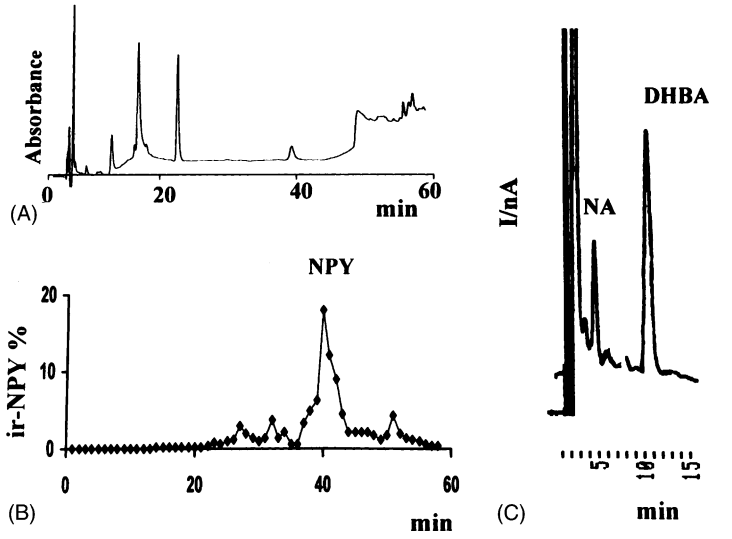

Fig. 3. Chromatographic identification of the outflow of ir-NPY and NA evoked by electrical stimulation of the perimesenteric arterial nerve terminals. (A) UV spectra of the characteristic absorbance chromatogram (220 nm, 64 mV) of a representative mesenteric perfusate. (B) RIA analysis of the above chromatogram expressed as a percentage of the total ir-NPY eluted from the column. Each fraction from the chromatogram was assayed by the NPY RIA. (C) The NA extracted from each perfusate sample was separated by HPLC and quantified by electrochemical detection. As an internal standard, 3,4-dihydroxybenzylamine (DHBA) was used.

only after treatment with 0.1–2 mg/kg reserpine (Fig. 4C), paralleling the reduction in NA release.

Pre-treatment with 0.1 and 1 mg/kg reserpine caused a significant increase in the basal ir-NPY values as compared to the non-treated rats (17.4 ± 3 ($n = 5$) and 19.2 ± 3.7 ($n = 5$) versus 8.8 ± 1.3 fmol ($n = 8$, $P < 0.05$), respectively). In contrast, parallel determinations of basal levels of NA showed that it was not detectable in the reserpinized rats (0.1 and 1 mg/kg).

3.4. Clonidine, an α_2-adrenergic agonist

To assess the role of the presynaptic α_2-adrenergic receptors in the control of co-transmitter release, mesenteries were perfused with varying concentrations of clonidine, a selective α_2-adrenergic agonist. Clonidine differentially reduced the electrically evoked release of ir-NPY and NA. Perfusion with 0.3 nM clonidine did not affect the release of luminally accessible ir-NPY nor NA elicited by the electrical depolarization. However, although 1 nM clonidine did not modify the outflow of ir-NPY, it reduced $60.3 \pm 12\%$ ($n = 4$, Fig. 5A) the NA outflow. In paired experiments, 1 nM clonidine did not change the basal outflow of co-transmitters prior to electrical stimulation. Clonidine 100 nM produced a maximal (70%) inhibition of the release of NA (2.9 ± 0.4 pmol (($n = 3$), $P < 0.001$) versus 6.6 ± 0.46, ($n = 16$) in control mesenteries), while it did not modify the outflow of the luminally accessible ir-NPY released (Fig. 5A). Perfusion with clonidine did not modify the rise in perfusion pressure elicited by the electrical depolarizations (Fig. 5B). Further experiments revealed that perfusion with 1–100 nM clonidine, without electrical depolarization, did not alter the basal release of ir-NPY nor NA.

Neither 10 nM clonidine nor 1 μM yohimbine modified the outflow of luminally accessible ir-NPY released by electrical depolarization (Fig. 6A). Likewise, the simultaneous perfusion of 10 nM clonidine plus 1 μM yohimbine did not modify the outflow of the luminally accessible ir-NPY (Fig. 6A). However, consistent with the results presented above, yohimbine, the α_2-adrenoceptor blocker, significantly increased the electrically evoked outflow of NA (19.7 ± 4.3 ($n = 3$) versus 6.6 ± 0.46 pmol (($n = 16$), $P < 0.01$, Fig. 6B). Furthermore, the simultaneous perfusion of 1 nM clonidine plus 1 μM yohimbine blocked the inhibitory effect of 1 nM clonidine (Fig. 6B). Yohimbine itself did not modify the release of NA prior to electrical depolarization. In these experiments, neither clonidine, nor yohimbine, nor the combination of clonidine plus yohimbine modified the rise in perfusion pressure evoked by electrical stimulation compared to untreated rat mesenteries (data not shown).

4. Discussion

Our finding that reserpine blunted the electrically evoked release of NA without modifying the release of ir-NPY and that clonidine, significantly reduces only the outflow of NA without modifying the release of ir-NPY, suggests that the release of these co-transmitters is regulated by different presynaptic mechanisms. The present results allow us to propose that the release of transmitters stored in the small vesicles is modulated differently from the release of transmitter(s) stored in the large vesicle. Furthermore, the

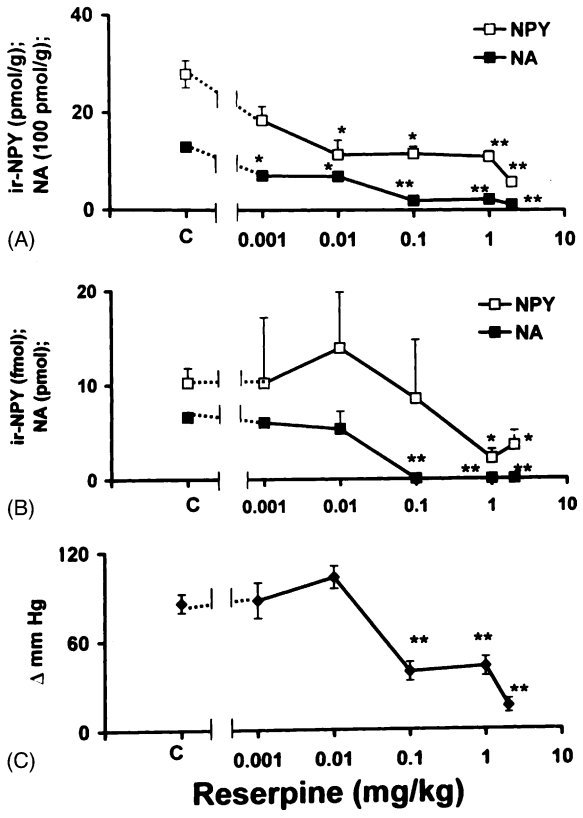

Fig. 4. Effect of different doses of reserpine on the ir-NPY and NA content, the electrically evoked release of ir-NPY and NA and the rise in perfusion pressure of the mesentery elicited by electrical depolarization. (A) Reduction in the tissue content of ir-NPY (□) and NA (■). (B) Decrease in the luminally accessible outflow of ir-NPY (□) and NA (■) elicited by electrical stimulation of the mesenteric artery perivascular nerve terminals. (C) Reduction in the rise in perfusion pressure elicited by the electrical stimulation of the nerve terminals. The letter C (control) in the abscissa of the panels refers to: panel A, the ir-NPY and NA content ($n = 15$ and 8, respectively) in control tissues, panel B, the outflow of ir-NPY and NA elicited by electrical stimulation in non-treated rats ($n = 12$ and 16, respectively) and panel C, the rise in the perfusion pressure evoked by the electrical depolarization in mesenteries from control rats ($n = 19$). The symbols (∗), $P < 0.05$; (∗∗), $P < 0.01$ (Dunnett's tables), symbols refer to the mean values, bars the S.E.M.

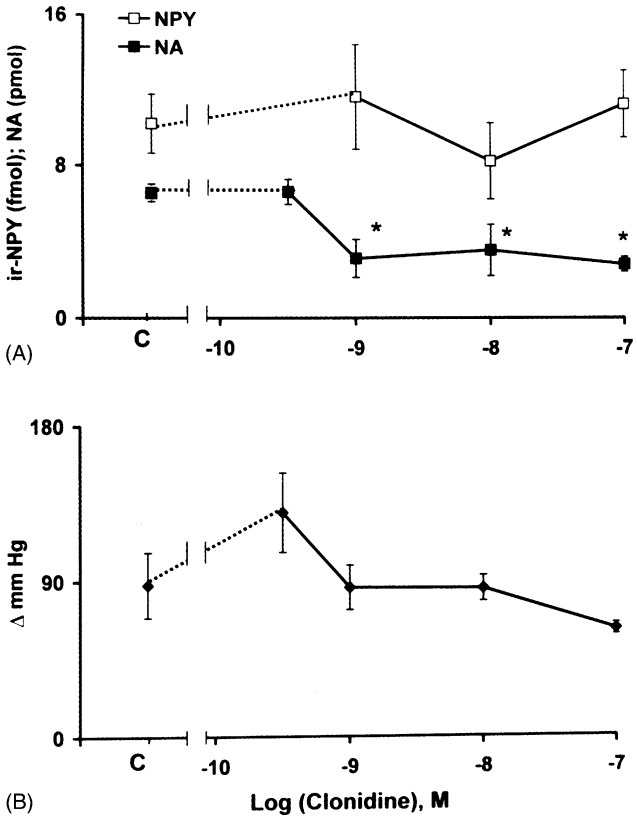

Fig. 5. Clonidine reduced the outflow of NA but not ir-NPY released evoked by electrical depolarization of the perivascular mesenteric artery nerve terminals. (A) Clonidine reduced in a concentration-dependent manner the electrically evoked outflow of NA (■) but not the release of ir-NPY (□); 4–7 different mesenteries were studied per clonidine concentration. Letter C in the abscissa indicates control mesenteries from rats perfused with buffer without clonidine ($n = 12$ for ir-NPY and $n = 16$ for NA). (B) Rise in perfusion pressure evoked by the 20-Hz train of electrical pulses in the rats perfused by varying concentrations of clonidine ($n = 4$–14). Letter C in the abscissa indicates the analysis of 19 control mesenteries. Symbols indicate the mean values; bars, S.E.M. The symbol (∗), $P < 0.05$, as compared to controls without clonidine (Dunnett's tables).

α_2-adrenoceptors modulate only the release of NA, while not affecting that of ir-NPY. Dunn et al. [10] also observed that in these same nerve terminals, α_2-presynaptic receptors differentially modulate the NA and ATP release, further supporting our notion of a differential presynaptic control of sympathetic co-transmitter release.

The immunohistochemical studies confirm the dense sympathetic innervation of the rat mesenteric bed originally described by Furness and Marshall [12] and the guinea pig inferior mesenteric vein by Smyth et al. [22]. The immunofluorescence for ir-NPY was similar than that for dopamine β-hydroxylase, and this fluorescence was blunted by the 6-hydroxydopamine treatment, confirming the sympathetic nature of NPY and dopamine β-hydroxylase. 6-Hydroxydopamine substantially destroyed the sympathetic nerve terminals, with the subsequent loss of co-transmitter content and release as previously described [25]. When the co-transmitter tissue content was determined in the perivascular nerve terminals, the ir-NPY and NA content in arteries is circa twice that found in the venules, consonant with the more intense arterial immunofluorescence. Regarding the ratio of NA/ir-NPY stored in blood vessels, our results are consistent with data from other sympathetic terminals [11] where the content of NA is much higher than that of ir-NPY. The detected cholinergic nerve endings emphasize that the perivascular nerve terminals surrounding the mesenteric artery contain other nerve terminals in addition to the sympathetic fibers. These terminals may be rich in peptides (CGRP or SP), and 5-HT, denoting their possible sensory nerve ending nature [29]. Positive staining for cholinesterase was not altered in the mesenteries of

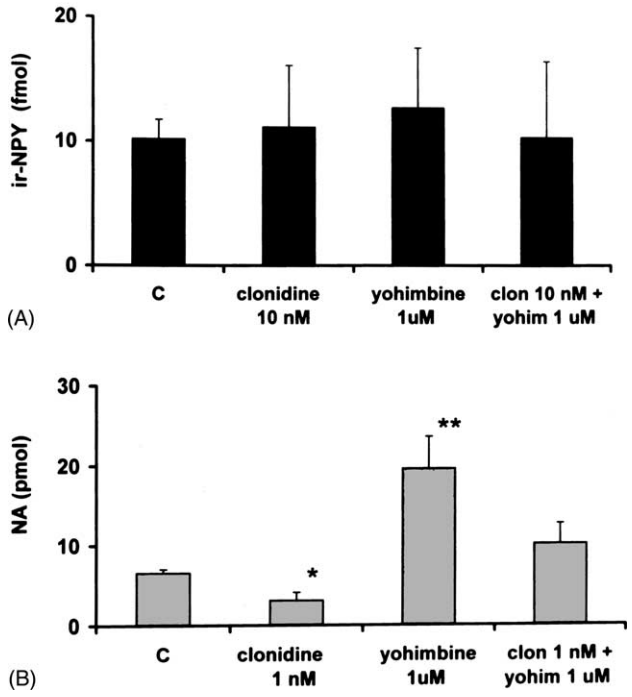

Fig. 6. α_2-Adrenoceptor antagonism modulates only the NA released by electrically evoked perivascular nerve terminal depolarization. (A) Neither yohimbine (1 μM, $n = 6$), nor clonidine (10 nM, $n = 12$), nor the combined application of both drugs ($n = 6$) modified the outflow of ir-NPY released by electrical depolarization. Separate mesenteries ($n = 12$) served as controls (C). (B) Yohimbine (1 μM, $n = 3$) increased three-fold the outflow of NA, while clonidine (1 nM, $n = 4$) halved the NA outflow. The joint application of both drugs did not modify the release of NA ($n = 4$). (C) It refers to the release of NA from 16 control mesenteries. Columns indicate the mean values; bars, the S.E.M. The symbols (∗), $P < 0.05$; (∗∗), $P < 0.01$ (Dunnett's tables).

6-hydroxydopamine pre-treated rats, confirming the selectivity of this drug to destroy sympathetic nerve terminals.

The depletion of vesicular NA induced by reserpine is known to be dose-, species-, and tissue-dependent [1]. These properties are likely related to the density of the sympathetic nerve terminals in each tissue, the anatomy of neuroeffector junctions, and the natural frequency of discharge of the nerve fibers intrinsic to each tissue. Reserpine reduces the vesicular content of NA by two mechanisms, only one of which affects NPY content. Reserpine depletes NA from the nerve terminals because it selectively blocks the vesicular catecholamine transporter, therefore preventing the storage of NA into the synaptic vesicles [13]. The second mechanism, which depletes both NPY and NA nerve terminal content, involves a secondary reflex involving transynaptic signal regulation, and leads to an increase in the ganglionic firing rate. This latter effect would induce an increase in the synthesis of NPY and NA precursors, as evidenced by increased mRNAs for pre-pro NPY and tyrosine hydroxylase. However, the augmented ganglionic firing rate does not allow a sufficient accumulation of these co-transmitters, resulting in a net reduction of ir-NPY and NA [14]. The reserpine-induced reduction in NA content and release was more marked than that of NPY, confirming the existence of the two separate mechanisms for co-transmitter storage and release. Furthermore, we found a significant increase in the basal release of ir-NPY only in the mesenteries of rats pre-treated with 0.1 and 1 mg/kg reserpine, a finding that is consistent with Hänze et al. [14]. These results provide evidence that the second mechanism operates only after a marked depletion of NA has occurred.

The rise in perfusion pressure seems to be due mostly to NA release; treatments that decreased the outflow of NA also decreased the increase in perfusion pressure. From a physiological point of view, it is of interest that the sympathetic nerve terminals can be depleted of NA by about 45% without loss in the NA release and with no changes in the functional response. A higher dose of reserpine (0.1 mg/kg) reduced even more the tissue co-transmitter content and abolished the NA release, with a concomitant loss of functional response. This reserpine dose did not modify the ir-NPY release. This result shows that the rise in perfusion pressure is mainly coupled to NA release.

Clonidine reduced only partially the electrically evoked output of NA, without significantly modifying the rise in perfusion pressure. Interestingly, yohimbine itself increased the electrically evoked release of NA, indicating that the NA released after electrical depolarization can control its further release, preventing its excessive outflow. The combined application of clonidine plus yohimbine demonstrated the expected antagonism, highlighting the physiological involvement of α_2-adrenoceptors in this control mechanism. As stated by Starke [24], the finding that a receptor antagonist has the opposing agonist effect is one of the three physiological and pharmacological criteria that establishes the functional identity of an autoreceptor mechanism. In support of the functional relevance of presynaptic α_2-adrenoceptors, the present data show that perfusion with 1 nM clonidine, a concentration that halves the release of NA, does not modify the rise of perfusion pressure elicited by nerve stimulation. This result indicates that a small fraction of NA is sufficient to ensure full neural transmission, and that NPY may facilitate the NA-induced vasomotor action, helping maintain the increase in perfusion pressure induced by electrical stimulation [4,8]. We should not ignore the fact that extracellular ATP, acting via P2X receptors, is also involved in sympathetic co-transmission and might also participate in the regulation of transmitter release [2,21].

Since ATP is avidly metabolized to adenosine by nucleotidases, released together with the sympathetic co-transmitters at neuroeffector junctions [27], the role of adenosine in co-transmitter release is of interest. Our finding that an adenosine A_2 receptor subtype differentially regulates the co-release of NPY and NA [9] substantiates our proposal that presynaptic receptors separately control sympathetic co-transmitter release. Furthermore, differential presynaptic regulation is also observed in other peripheral neuroeffector junctions [10,15,28], extending the validity of our

conclusions to a variety of sympathetic neurons, an indication that this is a physiologically relevant synaptic control mechanism.

In conclusion, we show that α_2-adrenoceptors presynaptically control the release of NA but not that of NPY. Other presynaptic receptors may regulate the release of NPY or ATP. The finding that functional presynaptic receptors participate in the control of co-transmitter release may be of clinical importance in the design and selection of therapeutic agents that separately modulate sympathetic co-transmission.

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References

[1] Allen JM, Schon F, Yeats JC, Kelly JS, Bloom SR. Effect of reserpine, phenoxybenzamine and cold stress on the neuropeptide Y content of the rat peripheral nervous system. Neuroscience 1986;19(4): 1251–4.

[2] Briones R, Miranda R, Huidobro-Toro JP. Distribution and pharmacology of P2X receptors in human blood vessels. Biol Res 2001;34:R53.

[3] Brock JA, Bridgewater M, Cunnane TA. β-Adrenoceptor mediated facilitation of noradrenaline and adenosine 5′-triphosphate release from sympathetic nerves supplying the rat tail artery. Br J Pharmacol 1997;120:769–76.

[4] Cortés V, Donoso MV, Brown N, Fanjul R, López C, Founier A, et al. Synergism between neuropeptide Y and norepinephrine highlights sympathetic cotransmission: studies in rat arterial mesenteric bed with neuropeptide Y, analogs and BIBP 3226. J Pharmacol Exp Ther 1999;289:1313–22.

[5] De León H, Bonhomme M, Thibault G, Garcia R. Localization of atrial natriuretic factor receptors in the mesenteric arterial bed. Comparison with angiotensin II end endothelin receptors. Circ Res 1995;77:64–72.

[6] De Potter WP, Dillen L, Annaert W, Tombeur K, Berghmans R, Coen EP. Evidence for the co-storage and co-release of neuropeptide Y and noradrenaline from large dense cored vesicles in sympathetic nerves of the bovine vas deferens. Synapse 1988;2:157–62.

[7] De Potter WP, Kurzawa R, Miserez B, Coen EP. Evidence against differential release of noradrenaline, neuropeptide Y, and dopamine-β-hydroxylase from adrenergic nerves in the isolated perfused sheep spleen. Synapse 1995;19:67–76.

[8] Donoso MV, Brown N, Carrasco C, Cortes V, Fournier A, Huidobro-Toro JP. Stimulation of the sympathetic perimesenteric arterial nerves release neuropeptide Y potentiating the vasomotor activity of noradrenaline: involvement of neuropeptide Y-Y_1 receptors. J Neurochem 1997;69:1048–59.

[9] Donoso MV, Huidobro-Toro JP. A2-adenosine presynaptic receptors modulate the release of noradrenaline (NE) but not neuropeptide Y (NPY). J Neurochem 2001;78:160.

[10] Dunn WR, Brock JA, Hardy TA. Electrochemical and electrophysiological characterization of neurotransmitter release from sympathetic nerves supplying rat mesenteric arteries. Br J Pharmacol 1999;128:174–80.

[11] Fried G, Lundberg JM, Theodorsson-Norheim E. Subcellular storage and axonal transport of neuropeptide Y (NPY) in relation to catecholamines in the cat. Acta Physiol Scand 1985;125:145–54.

[12] Furness JB, Marshall JM. Correlation of the directly observed responses of mesenteric vessels of the rat to nerve stimulation and noradrenaline with the distribution of adrenergic nerves. J Physiol 1974;239:75–88.

[13] Glowinski J, Iversen LL, Axelrod J. Storage and synthesis of norepinephrine in the reserpine treated rat brain. J Pharmacol Exp Ther 1966;151:385–99.

[14] Hänze J, Kummer W, Haass M, Lang RE. Effect of catecholamine depletion and denervation on neuropeptide Y (NPY) and tyrosine-hydroxilase (TH) mRNA levels in rat sympathetic ganglia. Exp Clin Endocrinol 1994;102:54–9.

[15] Huang Y, Lau CW, Chan NWK, Yao XQ, Chan FL. Prejunctionally mediated inhibition of neurotransmission by isoprenaline in rat vas deferens. Life Sci 1998;63:2107–13.

[16] Karnovsky MJ, Roots L. A direct-coloring thiocholine method for cholinesterase. J Histochem Cytochem 1964;12:219–22.

[17] Lundberg JMM. Pharmacology of cotransmission in the autonomic nervous system: integrative aspects on amines, neuropeptides, adenosine triphosphate, amino acids and nitric oxide. Pharmacol Rev 1996;48:113–78.

[18] Lundberg JM, Rudehill A, Sollevi A, Fried G, Wallin G. Co-release of neuropeptide Y and noradrenaline from pig spleen in vivo: importance of subcellular storage, nerve impulse frequency and pattern feedback regulation and resupply by axonal transport. Neuroscience 1989;28:475–86.

[19] Lundberg JM, Rudehill A, Sollevi A, Hamberger B. Evidence for co-transmitter role of neuropeptide Y in the pig spleen. Br J Pharmacol 1989;96:675–87.

[20] Miller RJ. Presynaptic receptors. Annu Rev Pharmacol Toxicol 1998;38:201–27.

[21] Racchi H, Irarrázabal MJ, Howard M, Morán S, Zalaquett R, Huidobro-Toro JP. Adenosine 5′-triphosphate and neuropeptide Y are co-transmitterrs in conjunction with noradrenaline in the human saphenous vein. Br J Pharmacol 1999;126:482–91.

[22] Smyth L, Bobalova J, Ward SM, Mutafova-Yambolieva VN. Neuropeptide Y is a cotransmitter with norepinephrine in guinea pig inferior mesenteric vein. Peptides 2000;21:835–43.

[23] Starke K. Presynaptic α-autoreceptors. Rev Physiol Biochem Pharmacol 1987;107:73–146.

[24] Starke K. Presynaptic autoreceptors in the third decade: focus on α_2-adrenoceptors. J Neurochem 2001;78:685–93.

[25] Thoenen H, Tranzer JP. The pharmacology of 6-hydroxydopamine. Annu Rev Pharmacol 1973;13:169–80.

[26] Todorov LD, Mihaylova-Todorova S, Craviso GL, Bjur RA, Westfall DP. Evidences for the differential release of the cotransmitters ATP and noradrenaline from sympathetic nerves of the guinea-pig vas deferens. J Physiol (London) 1996;496:731–48.

[27] Todorov LD, Mihaylova-Todorova S, Westfall TD, Sneddon P, Kennedy C, Bjur RA, et al. Neuronal release of soluble nucleotidases and their role in neurotransmitter inactivation. Nature 1997;387:76–9.

[28] Trachte GJ, Binder SB, Peach MJ. Indirect evidence for separate vesicular neuronal origins of norepinephrine and ATP in the rabbit vas deferens. Eur J Pharmacol 1989;164:425–33.

[29] Warton J, Gulbenkian S, Mulderry PK, Ghatei MA, McGregor GP, Bloom SR, et al. Calcitonin gene-related peptide (CGRP)-immunoreactive nerves in the cardiovascular system of the guinea pig and rat. J Auton Nerv Syst 1986;16:289–309.

[30] Zukowska-Grojec Z, Wahlestedt C. Origin and actions of neuropeptide Y in the cardiovascular system. In: Colmers WF, Wahlestedt C, editors. The biology of neuropeptide Y and related peptides. Totowa, NJ: Humana Press, 1993. p. 315–87.