Evidence for co-transmitter role of neuropeptide Y in the pig spleen

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1   The possible involvement of neuropeptide Y (NPY) in relation to noradrenaline (NA) and adenosine triphosphate (ATP) mechanisms in the sympathetic nervous control of the vascular tone and capsule contraction in the blood perfused pig spleen was investigated *in vivo*.

2   Local injections or infusions of NA, NPY and α-, β-methylene ATP (mATP) caused vasonconstriction (perfusion pressure increase) and capsule contraction (increased venous blood flow). ATP only evoked vasodilatation. NPY was about 50 fold more potent than NA as a vasoconstrictor, and the NPY response was more long-lasting. Reserpine treatment did not change the effects of NPY.

3   Electrical stimulation of the splenic nerves in control animals caused a frequency-dependent, guanethidine-sensitive output of both NPY-like immunoreactivity (-LI) and NA, suggesting co-release. The output of NPY-LI relative to NA was enhanced at high frequency stimulation. Furthermore, α-adrenoceptor blockade by phentolamine enhanced both the output of NPY-LI and NA while inhibition of the neuronal uptake of NA with desipramine reduced the low frequency stimulation-evoked overflow of NPY-LI. Preganglionic denervation did not change the output of NPY-LI or NA.

4   Reserpine treatment reduced both the splenic content of NA and NPY-LI. Preganglionic denervation inhibited the reserpine-induced depletion of the NPY content but not of NA in terminal areas. The stimulation-evoked NPY overflow was markedly enhanced, especially at low-frequency stimulation after reserpine, and the plasma levels of NPY-LI in the venous effluent were then in the nmolar range (i.e. where exogenous NPY induced vasoconstriction). The perfusion-pressure increase upon stimulation in reserpine-treated, preganglionically-denervated animals was highly correlated ($r = 0.91$) to the NPY overflow. The functional 0.5 Hz responses were reduced after reserpine, while at higher frequencies the functional effects were of similar magnitude to controls but longer-lasting.

5   Tyramine induced a release of NA but not of NPY-LI. Furthermore, the increase in perfusion pressure induced by tyramine was absent after reserpine.

6   After tachyphylaxis to the vasoconstrictor effects of mATP, the nerve stimulation-evoked, functional response as well as the NA and NPY-LI overflow were unchanged. After reserpine treatment, both the perfusion-pressure increase and NPY-LI overflow to nerve stimulation were reduced after mATP tachyphylaxis.

7   In conclusion, release of NPY rather than ATP may explain the long-lasting, non-adrenergic, splenic functional responses in reserpinized animals upon sympathetic stimulation. However, NA is most likely the main splenic transmitter when low-frequency stimulation is used under control conditions.

Neuropeptide Y (NPY), a peptide with 36 amino acid residues which was originally isolated from

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porcine brain (Tatemoto, 1982), co-exists with noradrenaline (NA) in postganglionic, sympathetic nerves in a variety of peripheral organs (Lundberg *et al.*, 1982) including the pig spleen (Lundberg *et al.*, 1988). Increasing evidence suggests that NPY is involved in sympathetic neurotransmission. NPY is

co-released with NA upon electrical stimulation of sympathetic nerves (Lundberg *et al.*, 1984a; 1986b,c) and during reflex activation under physiological conditions in man, such as physical exercise (Lundberg *et al.*, 1985b; Pernow *et al.*, 1986a). NPY binds to specific, high-affinity sites with receptor characteristics in membranes from the rat brain (Saria *et al.*, 1984) and the pig spleen (Lundberg *et al.*, 1988). NPY exerts prejunctional inhibitory actions on the nerve stimulation-evoked [^{3}H]-NA release from sympathetic nerves (Lundberg & Stjärne, 1984; Pernow *et al.*, 1986b) and induces vasoconstriction in a variety of vascular beds (Lundberg & Tatemoto, 1982; Edvinsson *et al.*, 1983; Lundberg *et al.*, 1984a,b) including the pig spleen (Lundberg *et al.*, 1986c; 1988).

Increasing evidence suggests that part of the vasoconstrictor response to sympathetic nerve stimulation in several organs *in vivo* (see Lundberg & Hökfelt, 1986) and isolated blood vessels *in vitro* (Burnstock & Kennedy, 1986) is resistant to pretreatment with α- and β-adrenoceptor-blocking agents. However, it has been difficult to exclude the possibility that high concentrations of locally released NA activate a separate class of adrenoceptors (see Neild & Hirst, 1984). Furthermore, very little of the sympathetic vasoconstrictor response to nerve stimulation in the spleen remains following blockade of NA mechanisms with α-adrenoceptor antagonists (Cubeddu *et al.*, 1974) or NA depletion due to reserpine treatment (Dixon *et al.*, 1979). Reserpine treatment in the guinea-pig and cat is associated with a concomitant depletion of NPY-LI in the heart and spleen through enhanced neuronal release (Lundberg *et al.*, 1984b; 1985c,d; 1986a,b). In contrast, adenosine triphosphate (ATP) mechanisms are uninfluenced by reserpine (Kirkpatrick & Burnstock, 1987).

In the present study the effects of adrenoceptor blocking agents and reserpine pretreatment on the sympathetic control of the pig spleen in relation to NA, NPY and ATP mechanisms have been investigated *in vivo*.

Methods

Fifty-two pigs (b.w. 25–35 kg) were premedicated with ketamine (25 mg kg^{-1}, i.m.) and atropine (0.05 mg kg^{-1}, i.m.). Anaesthesia was induced with sodium pentobarbitone (20 mg kg^{-1}, i.v.) and maintained by continuous infusion of this agent (5 mg kg^{-1} h^{-1}). The pigs were intubated and ventilated artificially and their body temperature was maintained at 37°C. Skeletal muscle paralysis was then induced with pancuronium (0.2 mg kg^{-1}, i.v.) and maintained with repeated doses every hour.

After heparinization (1000 iu kg^{-1}), one femoral artery was cannulated for recording of systemic arterial blood pressure, using a pressure transducer connected to a Grass polygraph. The femoral vein on this side was used for continuous infusions of Ringer solution or Macrodex to compensate for blood losses (see below). The contralateral femoral artery and vein were also cannulated to allow blood perfusion of the spleen. The spleen was dissected free from the mesentery and all accessory vessels (at caudal and cranial portions) were ligated. After ligation of the main splenic vessels close to the coeliac artery, the spleen was excised and placed in saline at +37°C. The main splenic artery was rapidly cannulated and constantly perfused with blood from the femoral artery at a rate of 25–35 ml min^{-1}, using a roller pump. The venous outflow from the spleen was directed back into the femoral vein. Perfusion pressure in the system on the arterial side (reflecting vascular resistance changes) was monitored continuously via an inlet close to the spleen, by use of a pressure transducer. The perfusion rate was adjusted to obtain a perfusion pressure of 75–80 mmHg. The blood flow in the splenic venous effluent (which is related to capsule contractions) was recorded in parallel with an electromagnetic flowmeter (Nycotron, Norway).

After perfusion of the spleen had been established (within 5 min of removal from the animal), the postganglionic nerves along the main splenic vessels were carefully dissected free and placed on platinum electrodes. A resting period of 30–45 min followed before any stimulations or infusions were performed. Electrical, nerve stimulations were delivered using a Grass S88 stimulator at 10 V, 5 ms with varying frequencies or patterns, giving a total of 240 shocks. Continuous stimulations at 0.5 Hz were performed for 8 min, at 2 Hz for 2 min and at 10 Hz for 24 s. In addition, intermittent stimulation with bursts at 20 Hz for 1 s every 10 s for 2 min (20b) were also used. Stimulation was carried out at 20 min intervals and, in an initial series of experiments, the reproducibility of the model was shown by repeating each type of stimulation twice in a random fashion (*n* = 4). After these four types of stimulation, a second series of 20 b stimulations was also applied after administration of different blocking agents. In controls, a further 5 stimulations at 20 b were carried out, and in reserpinized animals 3 or 4 stimulations (see Results).

Blood samples (5 ml during and 3 ml before and after stimulation) were collected in ice-chilled tubes containing EDTA (final concentration 10^{-2} M), from the venous outflow just before and after $\frac{2}{3}$ of the respective stimulation periods, as well as 30 s, 2, 5, 10 and 15 min after the stimulation. Arterial blood samples were obtained before and 5 and 15 min after

the stimulations. Samples were kept in an ice bath during the experiments and then centrifuged at 2000 r.p.m. at $+4°C$ for 15 min. Plasma samples (0.5 ml for each type of analysis) were collected and frozen at $-70°C$ for later determination of NPY-LI and endogenous catecholamines. Endogenous plasma NA and adrenaline (Ad) were determined by high performance liquid chromatography (h.p.l.c.) with electrochemical detection (Hallman et al., 1978). The content of NA was also quantified in biopsies from the spleen, kidney, right atrium of the heart and the quadriceps skeletal muscle obtained at the end of the experiments. Plasma NPY-LI was determined after acid ethanol extraction, by radioimmunoassay (RIA) with the NPY antiserum N1 (for details, see Theodorsson-Norheim et al., 1985). Bolton-Hunter labelled $[^{125}I]$-porcine NPY was used as a tracer. The content of NPY-LI in biopsies obtained at the end of the experiments from the spleen, kidney, the right atrium of the heart and from the quadriceps skeletal muscle was also determined after extraction with acetic acid at 95°C (see Theodorsson-Norheim et al., 1985). The output of NA from the spleen was corrected for local uptake by using corresponding uptake values for plasma Ad. The total output of NA and NPY-LI during and for 15 min immediately after nerve stimulation was calculated by multiplying the veno-arterial concentration differences by the plasma flow.

Local intra-arterial infusions or injections of drugs were made via an inlet close to the spleen. Substances were delivered via infusion at a rate of $200 \mu l \, min^{-1}$, by means of a micro-infusion pump. Since the amount infused and the arterial plasma flow were known, the doses of drugs have been given as estimated plasma concentrations. Bolus injections were made with 0.3 ml of the tested agent followed by 0.3 ml saline.

Reserpine pretreatment (1 mg kg^{-1}, i.v.) was performed with a single dose in an ear vein following premedication with ketamine (25 mg kg^{-1}, i.m.). Five different groups of reserpinized pigs were used. The first group ($n = 6$) was maintained under anaesthesia after reserpinization for 16 h before the start of experiments. The second group was given reserpine 24 h before the experiments and allowed to regain consciousness ($n = 5$). The third and fourth groups were subjected to sectioning of the left splanchnic nerve simultaneously with ($n = 8$) or one week before the reserpine injections 24 h before the experiments began ($n = 6$). These nerves have been shown in the cat to contribute with preganglionic fibres to about 80% of the noradrenergic sympathetic ganglionic cells in the coeliac ganglion innervating the spleen (Brown et al., 1961). The denervation procedure was performed after the same premedication, and under the same sodium-pentobarbitone anaesthesia and

skeletal muscle paralysis, as described above. A retroperitoneal approach was used via a left subcostal incision under sterile conditions. About 1 cm of the left splanchnic nerve was removed and the wound closed by sutures. Atropine (0.05 mg kg^{-1}, i.v.) and neostigmine (0.1 mg kg^{-1}, i.v.) were then given, and the tracheal tube was removed after spontaneous breathing had resumed. The fifth group of pigs was preganglionically denervated at the same time as reserpine was given, 72 h before the experiments. A separate control group of animals was preganglionically denervated without being reserpine treated 24 h before the experiments. The postoperative period was free from complications in all pigs. In the reserpine-treated and preganglionically denervated animals, the same series of stimulations were performed as in the control animals before and after local infusion of drugs. Injections of tyramine, NPY, NA, ATP and α-, β-methylene ATP (mATP) were made in four additional animals.

Drugs

Statistics

Data are presented as means ± s.e.mean. Statistical significance was calculated using Kruskal-Wallis analysis of variance with multiple comparisons (for further details, see Theodorsson-Norheim, 1986) or Student's t test.

Results

Effects of nerve stimulations in control animals

Under basal conditions plasma NA and Ad levels were about 50–60% higher in the arterial input than in the venous effluent suggesting uptake in the spleen (Table 1). No corresponding gradient for the plasma

Table 1   Mean arterial blood pressure (BP, mmHg) and plasma levels of noradrenaline (NA, nM), adrenaline (Ad, nM) and NPY-LI (pM) in aorta (a) and splenic vein (v) from anaesthetized control pigs, 1 h after pretreatment with desipramine (DMI; $0.5 \, mg \, kg^{-1}$, i.v.), 16 h after reserpine ($1 \, mg \, kg^{-1}$, i.v.) in pigs kept under anaesthesia and 24 h as well as 72 h after reserpine treatment of conscious animals; in one reserpine 24 h group and in the 72 h group the left splanchnic nerve was sectioned at the time of the reserpine injection

	BP (mmHg)	NA (nM)		Ad (nM)		NPY-LI (pM)	
		a	v	a	v	a	v
Control	106 ± 2	2.1 ± 0.3	1.3 ± 0.2	0.4 ± 0.1	0.2 ± 0.04	30 ± 4	36 ± 4
DMI	110 ± 3	1.3 ± 0.1	1.4 ± 0.2	0.5 ± 0.1	0.5 ± 0.1	26 ± 3	27 ± 4
Reserpine 16 h + anaesthesia	75 ± 4***	3.0 ± 0.5	1.6 ± 0.5	1.6 ± 0.2**	1.2 ± 0.2**	459 ± 26***	465 ± 25***
Reserpine 24 h	93 ± 4**	3.4 ± 0.5	1.2 ± 0.3	3.6 ± 1.2**	1.8 ± 0.6**	167 ± 48**	190 ± 46**
Reserpine 24 h + pregangl. denerv.	88 ± 3**	3.8 ± 0.4	1.7 ± 0.3	3.2 ± 0.9**	1.5 ± 0.4**	152 ± 37**	181 ± 38**
Reserpine 72 h + pregangl. denerv.	97 ± 3	2.6 ± 0.3	1.6 ± 0.2	1.50 ± 0.2**	1.1 ± 0.2**	29 ± 4	32 ± 5

Data represent means ± s.e.mean ($n = 6$–15). **$P < 0.01$, ***$P < 0.001$ compared to control, using Kruskal Wallis analysis of variance.

NPY-LI was observed. Pretreatment with desipramine (DMI) abolished the splenic NA and Ad gradients (Table 1). Electrical stimulation of the splenic nerves increased perfusion pressure (Figure 1), blood flow in the splenic venous effluent (not shown) and the output of NA and NPY-LI (Figure 2). The perfusion-pressure increase and output of NA and NPY-LI were frequency-dependent (Figure 2A) and reproducible. The blood-flow increase, however, diminished upon repeated stimulations due to the depletion of the stored blood cells. Therefore, this parameter for capsule contractions has only been quantified for the initial series of stimulations.

However, the blood flow information was used when calculating the total output of NA and NPY-LI.

Intermittent stimulation with 20 b caused a significantly larger perfusion-pressure increase (about 40%; $P < 0.01$) and enhancement (3 fold) of the output of NPY-LI relative to NA (1.6 fold), as compared to a continuous stimulation at 2 Hz, giving the same total number of impulses (Figure 2A). After DMI, the functional responses were enhanced following 20 b stimulations (by 30 and 40% regarding perfusion pressure and blood flow, respectively), in parallel with an increased NA overflow (by 60%). At 0.5 Hz the output of NPY was reduced significantly
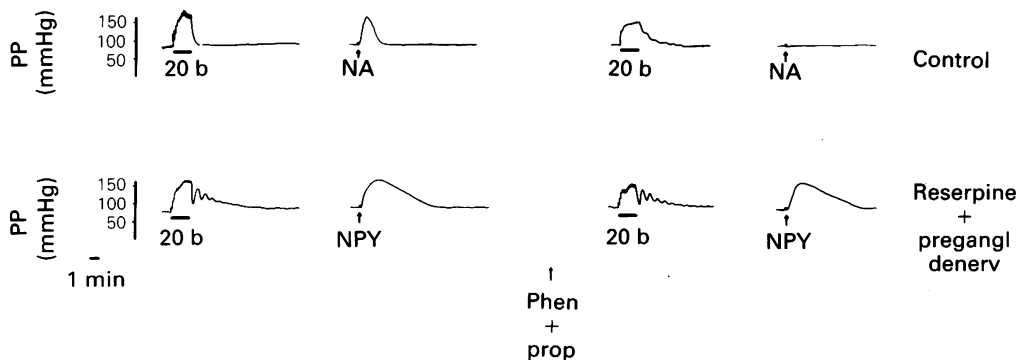
Figure 1   The effects of intermittent nerve stimulations with bursts at 20 Hz for 1 s every 10 s for 2 min (20b) or local bolus injections of noradrenaline (NA, 650 nmol) or neuropeptide Y (NPY, 2 nmol) on perfusion pressure (PP, mmHg) in the blood-perfused pig spleen *in vivo*. The effects under control conditions (top panel) are compared with the responses 24 h after reserpine ($1 \, mg \, kg^{-1}$, i.v.) treatment in animals where the spleen was denervated preganglionically one week prior to reserpine injections (lower panel). Local intra-arterial infusion of phentolamine (Phen) and propranolol (prop) (estimated local plasma concentrations of 5×10^{-5} and 10^{-6} M, respectively) was performed during the repeated stimulations or injections. Bar indicates time scale for 1 min.
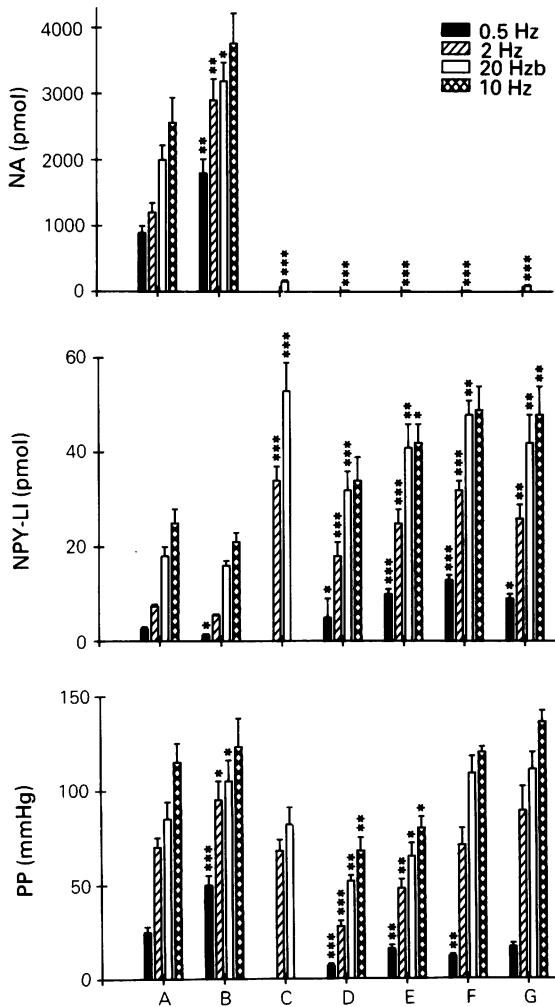
Figure 2 Perfusion-pressure increase (PP, mmHg) and the integrated output of neuropeptide Y-like immunoreactivity (NPY-LI) and noradrenaline (NA) (pmol) from the blood-perfused pig spleen *in vivo* during and the first 15 min after electrical stimulation of the splenic nerves with 0.5 Hz for 8 min, 2 Hz for 2 min and 10 Hz for 24 s, giving a total of 240 shocks. The effects of intermittent stimulation with bursts at 20 Hz for 1 s every 10 s for 2 min (20 b) are also illustrated. The responses are given as means (with s.e.mean shown by vertical bars) in (A) control animals (*n* = 5), (B) after pretreatment with desipramine (*n* = 5), (C) after resperpine treatment of pigs kept under anaesthesia for 16 h (*n* = 7), and 24 h (D, E, F) or 72 h (G) following resperine treatment of conscious pigs in intact (D) (*n* = 5) or preganglionically denervated preparations (1 week) (E), 24 h (F), or 72 h (G) (*n* = 6). *P < 0.05; **P < 0.01; ***P < 0.001 compared to responses in control animals, using Kruskal-Wallis analysis of variance with multiple comparisons.

(by 45%) compared to the response in controls (Figure 2B). Local pretreatment with phentolamine (estimated plasma concentration 5×10^{-5} M) caused a parallel enhancement of the output of both NPY-LI (from 20 ± 4 to 37 ± 4 pmol) and NA (from 1200 ± 150 to 2400 ± 450 pmol) upon 20 b stimulations.

Preganglionic denervation 24 h before the experiments did not change the functional responses or the overflow of NA and NPY-LI upon nerve stimulation (*n* = 4; not shown).

Effects of reserpine treatment

Sixteen and 24 h after reserpine treatment, the pigs had a lowered systemic arterial blood pressure (Table 1). Furthermore, plasma Ad and NPY-LI were markedly elevated (Table 1). Seventy-two hours after reserpine, blood pressure, plasma NA and NPY-LI were close to normal, while plasma Ad was still elevated (Table 1). After reserpine treatment of pigs kept either under anaesthesia or normal conscious conditions, the splenic NA content (Table 2) and the nerve stimulation-evoked output of NA from the spleen were markedly reduced (>90%) (Figure 2 C–G). In the pigs kept under anaesthesia after reserpine, the depletion of the NA content in the spleen (93%) and the reduction in the nerve stimulation-evoked output of NA (87%) were slightly less than in the animals which had been conscious for 24 h after the reserpine injection, where both parameters were reduced by 98–99% (Figure 2 C, D; Table 2). Preganglionic denervation for one week did not prevent the reserpine-induced depletion of NA in the spleen (Table 2). Three days after the injection of reserpine, about 10% of the initial NA content had recovered in the spleen (Table 2).

A marked depletion (>98%) of NA content 24 h after reserpine treatment also occurred in the right atrium of the heart (control: 15.4 ± 1.9 nmol g^{-1}, *n* = 11). Furthermore, the content of NA in the kidney (control: 1.8 ± 0.1 nmol g^{-1}, *n* = 10) and skeletal muscle (control: 0.12 ± 0.02 nmol g^{-1}, *n* = 6) were both reduced by 98% 24 h after the reserpinization of conscious pigs. In the adrenal gland, however, the content of NA was only reduced by 37% (from 5002 ± 489 nmol g^{-1} to 3165 ± 294 nmol g^{-1}; *n* = 6) and Ad levels by 77% (from 4323 ± 755 nmol g^{-1} to 983 ± 134 nmol g^{-1}; *n* = 6) 24 h after reserpine.

Reserpine treatment was also associated with a depletion of NPY-LI in the spleen, as revealed in biopsies obtained after the end of the stimulation experiments (Table 2). Furthermore, the contents of NPY-LI in the right atrium of the heart (control: 24.0 ± 3.0 pmol g^{-1}, *n* = 8), kidney (control: 1.8 ± 0.09 pmol g^{-1}, *n* = 5) and skeletal muscle

Table 2 The levels of neuropeptide Y-like immunoreactivity (NPY-LI, $pmol\,g^{-1}$) and noradrenaline (NA) ($nmol\,g^{-1}$) in the pig spleen from (1) controls, (2) animals where the spleen was preganglionically denervated 24 h earlier, (3) reserpine ($1\,mg\,kg^{-1}$, i.v.) pretreated pigs kept under anaesthesia for 16 h, (4) reserpine-pretreated pigs that were conscious for 24 h after the bolus injection, conscious reserpine-pretreated pigs where the spleen also was preganglionically denervated 1 week earlier (5) or simultaneously with the reserpine injection (6) and (7) conscious reserpine-treated pigs where the injection and preganglionic denervation were carried out 72 h before the experiment

	NPY-LI	%	NA	%
1 Control	19.1 ± 1.4 (12)	100	11.6 ± 1.0 (12)	100
2 Pregangl. denerv. 24 h	15.5 ± 0.8 (4)	81	10.7 ± 1.3 (4)	92
3 Reserpine 16 h + anaesthesia	11.7 ± 0.8 (6)	61**	0.8 ± 0.1 (7)	7***
4 Reserpine 24 h	5.3 ± 0.6 (5)	28***	0.1 ± 0.05 (5)	1***
5 Reserpine 24 h + pregangl. denerv. 1 week	10.1 ± 0.5 (6)	53***	0.1 ± 0.03 (6)	1***
6 Reserpine 24 h + pregangl. denerv. 24 h	15.5 ± 1.2 (8)	81	0.2 ± 0.04 (4)	2***
7 Reserpine 72 h + pregangl. denerv. 72 h	12.8 ± 1.5 (4)	67*	1.3 ± 0.3 (4)	11***

Data are given as means ± s.e.mean ($n = 4$–12). $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ denote significant changes from control, using Kruskal-Wallis analysis of variance with multiple comparisons.

(control: $0.12 \pm 0.02\,pmol\,g^{-1}$, $n = 4$) were reduced by 83, 78 and 42%, respectively 24 h after reserpine treatment of conscious animals. The adrenal in control pigs contained low levels of NPY-LI ($0.3 \pm 0.01\,pmol\,g^{-1}$) and after reserpine a slight reduction to $0.1 \pm 0.05\,pmol\,g^{-1}$ was observed ($n = 4$). When preganglionic denervation was combined with the reserpine treatment, a partial prevention of the NPY depletion in the spleen was observed with one week of denervation and close to a normal content after 24 h denervation (Table 2).

The perfusion-pressure increase in the spleen upon electrical nerve stimulation, when using high stimulation frequencies (20 b and 10 Hz) was well preserved in reserpine-treated animals (Figures 1, 2). Furthermore, the increase in blood flow in the splenic venous effluent was approximately 60% of the response to 20 b and 10 Hz in controls (not shown). However, the perfusion pressure response both to 0.5 and 2 Hz stimulations were reduced (by about 50%) in the non-denervated and 1 week-denervated groups, which had been conscious after the reserpine injection (Figure 2 D, F). The blood-flow increase upon 0.5 and 2 Hz stimulations was 40 and 50%, respectively, as compared to controls in the reserpine-treated animals where the spleen had been preganglionically denervated one week earlier. In the pigs that were preganglionically denervated simultaneously with reserpine injection, a major decrease in the perfusion-pressure response was observed only at 0.5 Hz (Figure 2 F). The perfusion-pressure responses in the animals, which had been preganglionically denervated and conscious after the reserpine injection were long-lasting compared to the effects under control conditions (Figure 1). Repeated stimulation in reserpinized animals caused a progressively diminishing functional response (not shown) (Table 3).

The output of NPY-LI upon nerve stimulation was markedly enhanced after reserpine treatment (Figure 2). Thus, after reserpine treatment performed simultaneously with or one week after a preganglionic denervation, the output of NPY-LI upon stimulation increased about 4, 3, 2 and 1.5 fold at 0.5, 2, 20 b and 10 Hz, respectively. There was a good correlation between the remaining perfusion-pressure responses and the overflow of NPY-LI (expressed as the total output ($r = 0.91$, $P < 0.001$) or as a maximal, veno-arterial concentration gradient ($r = 0.92$, $P < 0.001$) in the reserpinized animals (Figure 3a,b). The maximal increase in the plasma levels of NPY-LI in the splenic venous effluent upon 10 Hz stimulations in reserpinized animals was $1250\,pmol\,l^{-1}$. The output of NPY-LI, however, declined in the reserpinized animals upon repeated 20 b stimulation (not shown). Since a maximal output of around 60 pmol NPY-LI was recovered in the splenic venous effluent upon a 20 b stimulation for 2 min in reserpinized pigs, at least 3% of the total splenic NPY content (which was 1795 ± 132 pmol in control spleens; $n = 8$, corresponding to 8 µg of NPY) was released. Therefore, a considerable portion of the initial splenic NPY content is likely to have been depleted during the series of stimulations in the reserpinized animals before tissue samples were obtained (Table 2).

Pretreatment with propranolol did not influence the perfusion-pressure increase or the output of NPY-LI evoked by nerve stimulation in control or reserpinized pigs (Table 3). The perfusion-pressure increase to 20 b in control animals was prolonged and reduced by 35% after combined α- and β-adrenoceptor blockade, with phentolamine and propranolol (Figure 1), while the output of NPY-LI was enhanced as compared to controls (Table 3). In the conscious reserpine-treated animals, the

Table 3 The effects of local treatment with propranolol (10^{-6} M), phentolamine (5×10^{-5} M) plus propranolol (10^{-6} M) or guanethidine (3 mg kg^{-1}, i.v.) on nerve stimulation (20 b)-evoked perfusion-pressure increase and the output of NPY-LI from pig spleen

	Control		Reserpine Anaesthesia		Reserpine Concious + 24th pregangl. denerv.		Reserpine Concious + 72h pregangl. denerv.	
	Perfusion pressure increase	Output NPY-LI	Perfusion pressure increase	Output NPY-LI	Perfusion pressure increase	Output NPY-LI	Perfusion pressure increase	Output NPY-LI
Basal	100 ± 6	100 ± 15	100 ± 11	100 ± 11	100 ± 11	100 ± 12	100 ± 0	100 ± 15
Repeat	95 ± 8	107 ± 17	—	—	92 ± 2	92 ± 2	90 ± 2	85 ± 9
Propranolol	99 ± 4	89 ± 10	91 ± 7	108 ± 12	—	—	—	—
Phentolamine + propranolol	65 ± 4**	170 ± 27**	77 ± 3*	75 ± 15*	77 ± 6*	82 ± 4*	75 ± 10*	78 ± 6*
Guanethidine	3 ± 1***	4 ± 2***	—	—	3 ± 1***	2 ± 1***	—	—

Data are shown from controls, reserpinized animals that were simultaneously preganglionically denervated 24 or 72 h before the experiments. Values (means ± s.e.mean) have been expressed in percentage of the initial 20 b stimulation in each experiment ($n = 5$–6). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to the basal stimulation response in each group, using Kruskal-Wallis analysis of variance with multiple comparisons.
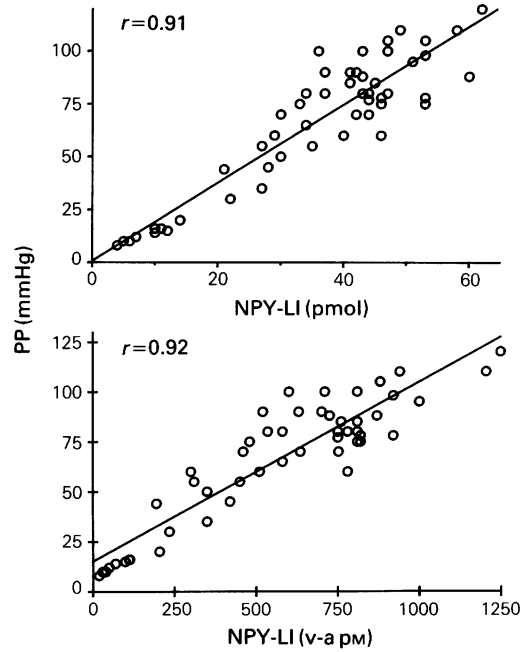

Figure 3 Correlation between perfusion-pressure increase (PP, mmHg), and (a) the recovered integrated output of neuropeptide Y-like immunoreactivity (NPY-LI) (pmol/stimulation) or (b) the maximal veno-arterial (v-a) plasma-concentration gradient of NPY-LI in the splenic venous effluent upon nerve stimulations with different frequencies of the blood-perfused spleen of reserpinized pigs (1 mg kg^{-1} given i.v. 24 h prior to the experiments in both control and preganglionically denervated animals).

perfusion-pressure responses (Figure 1) and the output of NPY-LI were also somewhat reduced after combined α- and β-adrenoceptor blockade (Table 3). Phentolamine and propranolol treatment *per se* increased the perfusion pressure, especially in the conscious reserpinized pigs (at 24 h, by 25 ± 4 mmHg and at 72 h, by 30 ± 5 mmHg). In the reserpinized animals kept under anaesthesia, pretreatment with phentolamine and propranolol prolonged and reduced the functional response in parallel with a slight reduction in the NPY output (Table 3). Guanethidine pretreatment virtually abolished the release of NPY-LI and the functional responses in control and reserpinized pigs (Table 3).

Effects of locally administered agents

Bolus injections of tyramine caused a long-lasting increase in the perfusion pressure in control animals. Simultaneously, there was an increased veno-arterial gradient of NA over the spleen, suggesting release
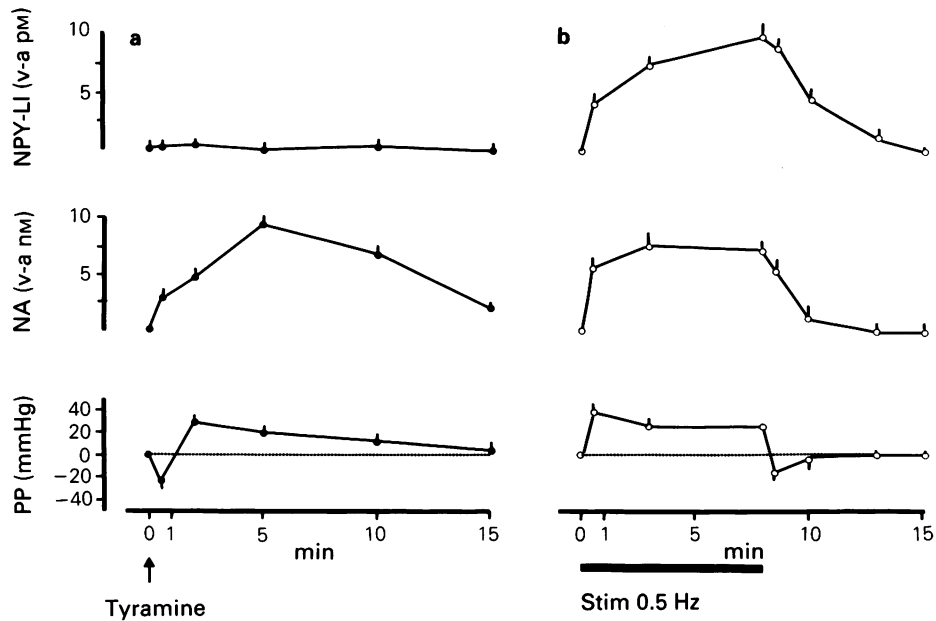
Figure 4 Effects upon local bolus injection of tyramine 4 μmol (arrow) or nerve stimulation with 0.5 Hz for 8 min on perfusion pressure (PP, mmHg) as well as veno-arterial (v-a) gradients of noradreneline (NA, nM) and neuropeptide Y-like immunoreactivity (NPY-LI, pM) from pig spleen. Values are given as means with s.e.mean shown by vertical lines, $n = 4$.

largely similar to the effect of the stimulation with 0.5 Hz, while no change in the NPY output occurred (Figure 4). In the reserpinized animals, no functional effects or NA release were evoked by tyramine (not shown).

NPY and NA were infused locally or given as bolus injections into control or reserpinized spleens which were not subjected to release experiments. NPY was about 50 fold more potent on a molar basis than NA in increasing the perfusion pressure with a threshold effect at 1000 pM. A very good recovery of the locally infused NPY was obtained in the splenic venous effluent as revealed by RIA. Thus, the levels of NPY-LI detected were 0.9 ± 0.1; 8.8 ± 1.2 and 85 ± 13 nM ($n = 4$) at the infusions, giving estimated local plasma concentrations of 1, 10 and 100 nM, respectively. The perfusion-pressure responses to bolus injections of NPY (Figure 1) or infusions of NPY were more long-lasting than those of NA and similar in control and reserpinized animals. Thus, the perfusion-pressure increases were 9 ± 1 mmHg vs. 11 ± 2 at 10^{-9} M NPY and 48 ± 5 vs. 52 ± 6 mmHg at 10^{-8} M NPY ($n = 4$) in controls and 24 h after reserpine combined with preganglionic denervation, respectively. Furthermore, both NA and NPY infusions were associated with an increased splenic venous blood flow, suggesting

capsule contraction of a comparable magnitude both in control and reserpinized animals (not shown).

The perfusion-pressure effects upon the infusion of 5×10^{-6} M NA (98 ± 5 mmHg) were abolished after pretreatment with phentolamine (5×10^{-5} M) and propranolol (10^{-6} M), while the response to NPY was not influenced by these adrenoceptor antagonists (not shown).

Local infusions of ATP (10^{-7} to 10^{-3} M) caused a dose-dependent reduction in the perfusion pressure (maximally by 70% at 10^{-3} M). Furthermore, the bolus injections of ATP (4 nmol to 4 μmol) also reduced the perfusion pressure (80% maximal reduction at 4 μmol ATP). The ATP analogue mATP caused a dose-dependent increase in the perfusion pressure and venous blood flow with a similar potency to NA. The mATP effect was not influenced by reserpine or the adrenoceptor antagonists. Upon continuous infusion of mATP (10^{-5} M), the initial marked perfusion-pressure increase was followed by a decline with time (Figure 5). Then, tachyphylaxis to the perfusion-pressure effects of mATP developed (Fig. 5). The maximal functional response to a 20 b stimulation as well as the output of NPY-LI and NA were not significantly changed upon mATP desensitization (Figures 5, 6). However, the perfusion-pressure response to nerve stimulation was reduced
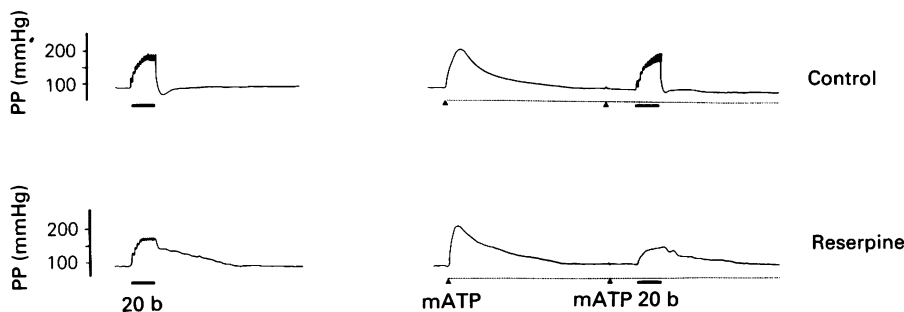
Figure 5 Effects of the infusion of mATP (10^{-5} M) on basal perfusion pressure (PP, mmHg) and the response to nerve stimulation (20 b for 2 min) in a control pig (top panel) and in an animal that was pretreated with reserpine combined with preganglionic denervation 24 h earlier (lower panel). A new mATP infusion (10^{-5} M) was started at the second right arrow head.
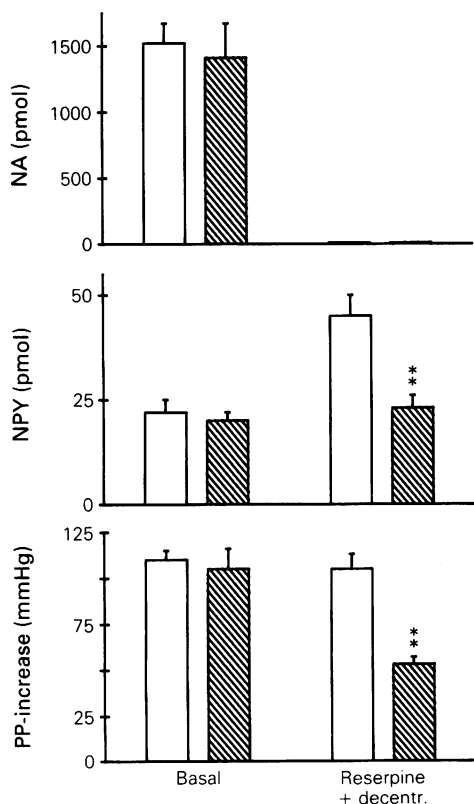
Figure 6 Summary of the effects of mATP, desensitization on the stimulation-evoked perfusion-pressure increase (PP, mmHg) as well as the integrated output of neuropeptide Y-like immunoreactivity (NPY-LI, pmol) and noradrenaline (NA, pmol) from the spleen of control and reserpinized pigs (24 h combined with preganglionic decentralization). Open columns, control; hatched columns, mATP. Data are given as means with s.e.mean shown as vertical bars ($n = 4$). **$P < 0.01$, comparing the responses before and after mATP tachyphylaxis.

by about 50% after mATP tachyphylaxis in reserpinized pigs (Figures 5, 6). The output of NPY-LI was then reduced in parallel (Figure 6).

Discussion

The present data show that in spite of pharmacological blockade of NA mechanisms by adrenoceptor antagonists and an almost total NA depletion after reserpine pretreatment, large functional responses (perfusion-pressure and blood-flow increases) remain in the pig spleen upon sympathetic nerve stimulation in vivo. In contrast, the functional effects of tyramine were absent in reserpine-treated animals. Thus, in the present in vivo model, no evidence was obtained that the tyramine response was non-adrenergic or associated with the release of NPY-LI, which is in apparent contrast to the in vitro data from the rat vas deferens reported by Cheng & Shen, 1987. It should be emphasized that adrenergic transmission has been reported to occur with small amounts of NA in reserpinized animals (see Andén & Henning, 1966; Antonaccio & Smith, 1974). However, the largely parallel reduction of the functional effects as well as the NPY overflow after phentolamine and propranolol pretreatment in the reserpinized animals indicate that the 10% of the initial content of NA remaining is not sufficient to contribute as a main splenic transmitter. Furthermore, the enhancement of the stimulation-evoked NPY overflow after reserpine was similar regardless of whether the NA content was 10 or 1% of control.

The prolongation of the nerve-stimulation responses by phentolamine and propranolol as well as the perfusion-pressure increase by these agents per se in reserpinized animals may be related to the inhibition of a vasodilator tone due to the high Ad levels in arterial plasma. Thus, the reserpine dose used, only partially depleted the adrenal catecholamines. Propranolol treatment in control animals did not

influence the basal or stimulation-evoked perfusion-pressure increase, suggesting that the Ad levels were then too low to influence the vascular tone and that neuronally released NA does not activate the vascular β-adrenoceptors to any major extent. The prolongation of the perfusion-pressure responses after adrenoceptor blockade in control animals (see also Lundberg & Tatemoto, 1982) and after reserpine treatment may also be related to the enhanced overflow of NPY-LI. In addition, metabolic production of vasodilator factors, such as adenosine, via adrenergic mechanisms (see Fredholm, 1976) are likely to contribute to the relatively rapid decline in the perfusion-pressure response observed after the stimulation under control conditions.

The absence of detectable NA release and the 99% depletion of NA content in the tissue 24 h after reserpine treatment of conscious animals suggest that activation of classical or novel adrenoceptors (see Nield & Hirst, 1984) is unlikely to account for the observed remaining splenic functional effects under these conditions. Since bolus injections of NPY mimicked these long-lasting functional responses and NPY is released upon splenic nerve stimulation, as indicated by increased overflow (see also Lundberg et al., 1984a; 1986c) this peptide is a possible candidate for the mediation of these apparently 'non-adrenergic' effects. Furthermore, the plasma levels of NPY-LI in the splenic venous effluent upon nerve stimulation at 20 b and 10 Hz after reserpine treatment were in the same range (nM) as the arterial plasma levels during the infusions of exogeneous NPY which are associated with vasoconstriction (see also Lundberg et al., 1986c; Rudehill et al., 1987). In addition, it is likely that the local concentrations of NPY-LI close to the nerve terminals after release are considerably higher than the spillover recovered in the venous effluent. A close relationship between NPY and the functional responses in reserpinized pigs was further supported by the excellent correlation ($r = 0.91$) between the remaining perfusion-pressure responses upon splenic nerve stimulation and the recovered output of NPY-LI in the splenic venous effluent. When using a low continuous stimulation frequency (0.5 Hz), most of the functional effects under control conditions are, however, likely to be related to NA, considering the relatively modest functional responses present in spite of a large increase in the corresponding output of NPY-LI in the reserpinized animals.

The release of NPY-LI upon low-frequency stimulation was slightly reduced when neuronal uptake was inhibited by DMI and enhanced after the administration of the α-adrenoceptor antagonist phentolamine. Furthermore, the NPY output was to an even greater extent enhanced when NA was depleted after reserpine pretreatment. This suggests

that NA exerts an inhibitory, prejunctional feedback influence on the release of NPY-LI via α-receptors, especially at low frequency stimulation (see also Lundberg et al., 1984a; 1986c). The release of NA was also enhanced after local phentolamine treatment in accordance with the view that the NA release from the spleen is subjected to autoregulation by prejunctional α-adrenoceptors (see Cubeddu et al., 1974).

Guanethidine pretreatment abolished the release of NPY-LI and perfusion-pressure increase upon splenic nerve stimulation in both control and reserpinized pigs. This observation is in accordance with the view that the NPY release originated from adrenergic nerve terminals capable of taking up guanethidine (see Boura & Green, 1965).

It has recently been shown that pretreatment with high doses of reserpine also depletes NPY-LI from certain sympathetic nerve terminals via a mechanism likely to involve neurogenic activation and release of the peptide in an excess of resupply by axonal transport (Lundberg et al., 1985c,d; 1986a). The content of NPY-LI in the pig spleen was in the present study also reduced after reserpine pretreatment, as determined after the stimulations. Part of this reduction, however, may be attributed to the stimulation-evoked release and depletion of terminal stores during the experiments, considering slow replacement of NPY by axonal transport.

The enhanced, stimulation-evoked NPY output from the pig spleen after reserpine treatment is in agreement with the increased release (see Dixon et al., 1979) and depletion of the splenic contents (Arnaiz et al., 1978) of dopamine-β-hydroxylase, another component of storage granules. This suggests that the increased impulse activity, which has been reported to be present in the sympathetic nerves of reserpine-treated animals (see Heusler, 1974), as well as the present observations of an enhanced release of NPY-LI per nerve impulse, created a situation in the non-denervated animals where resupply of the peptide was inadequate. Part of the increased release of NPY-LI after reserpine may be related to a loss of the inhibitory prejunctional feedback by NA, although other actions of reserpine may also contribute (see Cubeddu & Weiner, 1975).

Increasing evidence based on observations from in vitro experiments on isolated blood vessels (Kugelgen & Starke, 1985; Kennedy et al., 1986; Burnstock & Warland, 1987; Muramatsu, 1987) and vas deferens (Fedan et al., 1981; Sneddon et al., 1982; Stjärne & Åstrand, 1985), suggests that ATP is a co-transmitter with NA in the electrical and/or contractile responses of smooth muscle cells evoked by sympathetic nerve stimulation. It seems clear, however, that the proposed ATP-mediated com-

ponent of the smooth muscle response of isolated blood vessels is a rapid short-lasting contraction where the ATP contribution relative to NA is inversely related to stimulation frequency (i.e. relatively largest at low stimulation frequencies) (Kennedy et al., 1986; Burnstock & Warland, 1987). In contrast, the remaining nerve stimulation-evoked responses in the pig spleen after reserpine were slowly developing, long-lasting and were most pronounced at high frequencies. The depolarization-evoked ATP release is considered to be unaffected by reserpine treatment (Katsuragi & Su, 1981; Kirkpatrick & Burnstock, 1987; see Burnstock & Kennedy, 1986), and the non-adrenergic, presumably ATP-mediated, vasoconstrictor effects are also uninfluenced by reserpine (Muramatsu, 1987). This is not in line with the decline of the functional responses upon repeated stimulation in the spleen of reserpinized animals. It has, in fact, not been possible to provide conclusive evidence that nerve stimulation is associated with release of ATP from the perfused spleen (Stjärne et al., 1970) or a depletion of the splenic storage-vesicle content of ATP (Blaschke & Uvnäs, 1979). In addition, the reserpine-induced depletion of NA in the rat heart was not accompanied by any corresponding change in the vesicular content of ATP (Potter & Axelrod, 1963).

In the pig-spleen model, infusion of the metabolically stable ATP analogue mATP, which acts on P_2-purinoceptors (see Burnstock & Kennedy, 1986), caused a perfusion-pressure increase in vivo, suggesting vasoconstriction (see Delbro et al., 1985; Burnstock & Kennedy, 1986) both in control, in reserpinized animals and after adrenoceptor blockade. However, ATP only induced vasodilatation even in very high doses, which may be related to P_2-purinoceptor activation on endothelial cells (see Furchgott, 1981) and/or rapid degradation to adenosine, which is a potent vasodilator agent acting on P_1-purinoceptors on the vascular smooth muscle cells (see Burnstock & Kennedy, 1986).

The functional perfusion-pressure increase in the pig spleen to stimulation under control conditions was not influenced by mATP tachyphylaxis in spite of short bursts of impulses being used that would have favoured the putative ATP mechanisms (see Burnstock & Kennedy, 1986). In accordance with earlier reports, the nerve stimulation-evoked release of NA (Kugelgen & Starke, 1985; Stjärne & Åstrand, 1985) and NPY-LI (present results) was not reduced

upon mATP tachyphylaxis in control animals. When tachyphylaxis had developed to the perfusion-pressure response of mATP after reserpine treatment, the maximal perfusion-pressure effects upon splenic nerve stimulation were reduced. Then, the output of NPY-LI was inhibited in parallel. This suggests that mATP under these conditions caused an inhibitory effect on NPY release, via the activation of prejunctional P_2-purinoceptors (Fujioka & Cheung, 1987) or alternatively by some other pharmacological effect, such as the formation of adenosine or prostaglandins (Needleman et al., 1974; Frew & Baer, 1979) due to prolonged vasoconstriction. Furthermore, stable ATP analogues have been reported to depress the stimulation-evoked acetylcholine release from the intestine (Wiklund et al., 1985). The inhibitory effect of mATP on sympathetic transmission in the pig spleen seemed to be absent under control conditions, possibly due to a dominance of the powerful α-adrenergic feed-back regulation. Earlier findings using the pithed rat model in vivo have also shown that stimulation of the sympathetic outflow under control conditions results in a vasopressor response, which is not influenced by mATP tachyphylaxis (Flavahan et al., 1985). In contrast, the vasopressor effect to nerve stimulation after reserpine is reduced by mATP in agreement with the present data from the pig spleen. The influence of mATP on NPY release in reserpinized animals has then to be taken into account.

In conclusion, reserpine treatment combined with preganglionic denervation creates a situation where the high-frequency, stimulation-evoked, long-lasting, functional responses are likely to depend on the release of a non-adrenergic mediator, such as NPY rather than ATP. However, the possible importance of NPY compared to the classical transmitter NA (Peart, 1949) in the sympathetic control of the pig spleen during physiological conditions remains to be established by use of specific antagonists.

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