Dissociation of Potentiation of Leu\textsuperscript{31} Pro\textsuperscript{34} Neuropeptide Y on Adrenergic and Purinergic Transmission in Isolated Canine Splenic Artery

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Received January 31, 2000    Accepted March 21, 2000

ABSTRACT—The present study observed the effects of an activation of neuropeptide Y (NPY) Y\textsubscript{1} receptors on adrenergic and purinergic components of double-peaked vasoconstrictor responses to periartrial nerve stimulation in the isolated, perfused canine splenic arteries. The results showed that 3–30 nM Leu\textsuperscript{31} Pro\textsuperscript{34} neuropeptide Y (LP-NPY) produced a dose-dependent potentiation of double-peaked vasoconstrictor responses to trains of 30-s pulses at 1, 4 or 10 Hz of stimulation. The potentiation of LP-NPY of the nerve-stimulated vasoconstrictions were completely inhibited by subsequent blockade of \(\alpha\)-adrenoceptors or Y\textsubscript{1} receptors with 0.1 \(\mu\)M prazosin or with 1 \(\mu\)M BIBP 3226 ((R)-N\textsuperscript{2}-(diphenylacetyl)-N\textsuperscript{-}(4-hydroxyphenyl)methyl)-arginamide), respectively. The remaining responses in the presence of LP-NPY and prazosin were abolished by P2X receptor desensitization with 1 \(\mu\)M \(\alpha\),\(\beta\)-methylene ATP. Moreover, 30 nM LP-NPY failed to modify the vasoconstrictor responses to nerve stimulation after treatment with prazosin. A subsequent administration of \(\alpha\),\(\beta\)-methylene ATP completely suppressed the remaining responses after prazosin and LP-NPY. The vasoconstrictions induced by 0.003–1 nmol noradrenaline and 0.003–1 \(\mu\)mol ATP were slightly, but not significantly enhanced by 30 nM LP-NPY. The observations indicated that activation of postjunctional NPY Y\textsubscript{1} receptors may have an important role in the modulation of adrenergic rather than purinergic transmission of the sympathetic co-transmission.

Keywords: Leu\textsuperscript{31} Pro\textsuperscript{34} neuropeptide Y, NPY Y\textsubscript{1} receptor antagonist, BIBP 3226, Sympathetic nerve stimulation, Splenic artery

There is some evidence to support the idea that neuropeptide Y (NPY) may act as one of the co-transmitters with noradrenaline (NA) and adenosine 5' triphosphate (ATP) in the peripheral sympathetic nerve system, as reviewed by Lundberg (1). It is well known that the main functional roles of NPY in the sympathetic nerve are likely to be presynaptically mediated inhibition and postjunctionally mediated potentiation of sympathetic transmission (1). Previous investigations obtained with the sympathetic nerve innervating tissues have shown that NPY may potentiate the postjunctional adrenergic contractile responses to nerve stimulation and exogenous administration of NA (2, 3). Further observations showed that NPY also potentiated the postjunctional purinergic contractile responses to nerve stimulation (4, 5) and exogenous ATP (4, 6–8). It seems clear that the postjunctional synergistic effects of NPY is likely due to activation of NPY Y\textsubscript{1} receptors (6, 8, 9). The activation of NPY Y\textsubscript{1} receptors may have an important functional role under the physiological condition, since it is reported that endogenous NPY released from sympathetic nerves in the pig spleen mainly activates NPY Y\textsubscript{1} receptors (10). Donoso et al. (9) also reported that NPY released from the perimesenteric arterial sympathetic nerves acts via an activation of NPY Y\textsubscript{1} receptors, as the mediator responsible for the potentiation of NA on perfusion pressure in the isolated rat mesenteric bed. Leu\textsuperscript{31} Pro\textsuperscript{34} neuropeptide Y (LP-NPY), a selective NPY Y\textsubscript{1}-receptor agonist (11) appears to produce a similar postjunctional effect to NPY at the vascular sympathetic neuroeffector junction (6–8, 12). In contrast to NPY, LP-NPY causes no prejunctional inhibition of transmitter release in the sympathetically inervated epididymis of the guinea pig (12). Recently, a potent and selective non-peptide Y\textsubscript{1} receptor antagonist, BIBP 3226, was developed (13). Using BIBP 3226 as a functional Y\textsubscript{1}-receptor antagonist, it was demonstrated that BIBP 3226 antagonizes the postjunctional vasoconstrictions induced by NPY and LP-NPY (8, 10, 14). It has been demonstrated that vasoconstrictor responses to the perivascular
nerve stimulation in the canine splenic artery are mediated by the release of sympathetic co-transmitters NA and ATP (15–17). As demonstrated recently, in the canine splenic artery, periartrial electrical nerve stimulation readily induced a double-peaked vasconstriction, where the first phase response might contain mainly a purinergic component, and the second responses, mostly an adrenergic component (18, 19). Recent results obtained with the canine splenic artery suggested that there might be some separate modulatory mechanisms for the release of co-transmitters, NA and ATP. A small dose of tetrodotoxin selectively inhibited the second-peaked responses but not the first-peaked ones (20). The treatment with small doses of guanethidine also selectively inhibited the second-peaked responses (21). Treatment with an uptake blocker, imipramine, induced a potentiation of the second-peaked response but not the first one (19). On the other hand, several days of cold storage caused a depression of the first-peaked response much markedly more than the second-peaked response (22, 23).

In the present study, we wished to determine the effects of LP-NPY and BIBP 3226 on double-peaked vasconstrictor responses to periartrial nerve stimulation in the canine splenic artery and to characterize the modulation of an activation of NPY Y1 receptors in purinergic and adrenergic transmission in the peripheral sympathetic nerve system.

MATERIALS AND METHODS

Arterial preparations

Mongrel dogs of either sex, weighing 9 to 15 kg, were anesthetized with sodium pentobarbital (30 mg/kg, i.v.). After treatment with sodium heparin (200 units/kg, i.v.), the dogs were killed by rapid exsanguination from the right femoral artery. The arterial main branches of the splenic artery were isolated, and side branches of the artery were tied with silk threads. The artery (1 – 1.2 mm in outer diameter) was cut into segments (15 – 20 mm in length), and only four segments were obtained from each splenic artery. Each segment was cannulated and set up for perfusion as described previously (24, 25). Briefly, a stainless steel cannula was inserted into the arterial segment from the distal to the proximal end. The proximal portion of the segment was fixed to the distal portion of a needle type cannula with silk threads. The cannula was 3 – 4 cm in length and 0.8 – 1.0 mm in an outer diameter and had small side holes 5 mm from the distal sealed end. The cannulated arterial segment was placed in a cup-shaped glass bath and was perfused by a roller pump (Tokyo Rikakikai, Tokyo) with Krebs-Henseleit solution gassed with 95% O2 and 5% CO2. The solution contained 118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 25 mM NaHCO3 and 10 mM glucose. The flow rate was kept at approximately 2 ml/min. The perfusion pressure was continuously measured with an electric manometer (MPE-0.5A; Nihon Kohden, Tokyo) and recorded with a rectigraph (WT-685G, Nihon Kohden). After a stabilization period of 60 min, the preparation was removed from the bath solution and fixed in a horizontal position. The preparation was perfused at a constant flow rate during the experiment. The basal perfusion pressure was within 40 – 80 mmHg.

For electrical stimulation of the periartrial sympathetic nerve terminals, two platinum electrodes were placed on the extraluminal side of the arterial wall. Electrical stimulation was delivered by an electric stimulator (SEN-7203, Nihon Kohden) using 30-s trains of pulses at 10-V amplitude, 1-ms pulse duration and a frequency range of 1, 4 and 10 Hz. The organ bath was sealed with plastic film to maintain the preparation at 37°C. Ten-minute intervals between electrical stimulation periods were needed to obtain reproducible responses. The intervals between frequency-response curves were 60 min. The preparations were incubated for 60 min with prazosin, αβ-methylene ATP (αβ-m ATP) and BIBP 3226 or for 30 min with LP-NPY before the next response curves were made for electrical stimulation. The drug solutions of NA or ATP were administered into the rubber tubing close to the cannula in a volume of 0.01 – 0.03 ml by means of microinjectors.

Drugs

Drugs used were lithium αβ-methylene adenosine 5'-triphosphate and Leu31 Pro34 neuropeptide Y (human, rat) (Research Biochemicals International, Natick MA, USA); prazosin hydrochloride, disodium adenosine 5'-triphosphate, tetrodotoxin (Sigma, St. Louis, MO, USA); dl-noradrenaline hydrochloride (Sankyo, Tokyo). BIBP 3226 ((R)-N2-(diphenylacetyl)-N-[4-hydroxyphenyl)methyl]-argininamide) was kindly provided by Boehringer Ingelheim Pharma KG (Biberach, Germany). Tetrodotoxin and Leu31 Pro34 neuropeptide Y were dissolved in 0.5% (w/v) bovine serum albumin in distilled water. Other drugs were dissolved in physiological saline before the start of the experiment. The stock solutions were kept at −20°C until used.

Statistical analysis

Vasoconstrictor responses to electrical stimulation are expressed as the maximal changes in perfusion pressure (mmHg) from their control levels. The data are shown as the mean ± S.E.M. An analysis of variance with Bonferroni's test was used for statistical analysis of multiple comparisons of data. P values <0.05 was considered statistically significant.
RESULTS

Vascular responses to periarterial electrical nerve stimulation

The periarterial electrical nerve stimulation (30-s trains of pulses) induced a double-peaked vasoconstriction (2 phases of vasoconstriction) in the isolated and perfused canine splenic artery in a frequency-related manner (1, 4 and 10 Hz) and usually separated by an intervening dip in the increasing perfusion pressure (Figs. 1A, 3A and 5A) as reported previously (18). The first peak of vasoconstriction was reached within 8–12 s and the second peak, within 30–35 s after the start of electrical stimulation as shown in Figs. 1A, 3A and 5A. The double-peaked vasoconstrictions following nerve stimulation were completely inhibited by tetrodotoxin (30 nM) (data not shown, n = 4).

Effects of prazosin and αβ-m ATP on the LP-NPY-induced potentiation of vasoconstrictor responses to nerve stimulation

Intraluminal application of 3 nM LP-NPY did not produce any significant vascular contractile response, but 30 nM LP-NPY induced a slight vasoconstriction by itself (12 ± 11.5 mmHg, n = 26). After the treatment with 3–30 nM LP-NPY, the double-peaked constriction was readily potentiated. Figure 1 shows an original tracing of vascular contractile force from typical experiments showing the effects of prazosin and αβ-m ATP on the LP-NPY-induced potentiation of vasoconstrictor responses to nerve stimulation. As shown in Fig. 2A, a lower concentration of LP-

![Diagram](image)

Fig. 1. Double-peaked vasoconstrictor responses to periarterial nerve electrical stimulation (ES) (A) and the effects of LP-NPY (B, C), as well as effects of prazosin and αβ-m ATP, on the 30 nM LP-NPY-potentiated responses (D, E) in an isolated, perfused canine splenic artery. The vessel was electrically stimulated by 30-s trains of pulses at 10-V amplitude and 1-ms pulse duration, with a frequency of 1, 4 or 10 Hz.
NPY (3 nM) slightly, but not significantly potentiated the first phase vasoconstrictions at any frequencies used. On the other hand, it significantly enhanced the second phase responses at lower frequencies (1 and 4 Hz) and slightly potentiated the second one at a high frequency (10 Hz) (non significant). A tenfold increased concentration of LP-NPY (30 nM) markedly potentiated both the first responses and the second responses at any frequencies used (Fig. 2). A subsequent treatment with prazosin (0.1 μM) completely inhibited the potentiation of LP-NPY of the vasoconstrictor responses following nerve stimulation (Fig. 2). The remaining responses in the presence of LP-NPY and prazosin were suppressed by subsequent application of 1 μM aβ-m ATP (Fig. 2).

**Effects of BIBP 3226 on the LP-NPY-induced potentiation of vasoconstrictor responses to nerve stimulation**

Figure 3 shows an original tracing of vascular contractile response from typical experiments, showing blocking effects of BIBP 3226 on the LP-NPY-induced potentiation of vasoconstrictor responses to nerve stimulation. As shown in Fig. 4, LP-NPY at dose of 30 nM significantly potentiated both the first and the second responses to nerve stimulation at 1, 4 and 10 Hz. A subsequent treatment with BIBP 3226 (1 μM) completely inhibited the potentiation of LP-NPY (Fig. 4). Moreover, a subsequent treatment with 1 μM BIBP 3226 produced an additional inhibition on the second peaked responses at low frequencies (1 and 4 Hz), and at a high frequency (10 Hz), BIBP 3226 caused a slight but significant inhibition on both the first and second
peaked vasoconstrictions (Fig. 4).

Effects of LP-NPY on the vasoconstrictor responses to nerve stimulation in the presence of 0.1 μM prazosin

The treatment with 0.1 μM prazosin abolished the vasoconstrictor responses to exogenous NA (0.003–1 nmol), but did not influence the contractile responses to ATP (0.003–1 μmol). On the other hand, the perfusion with αβ-m ATP (1 μM) blocked the contractile responses to ATP, but not those to NA as reported previously (18). Figure 5 shows an original tracing of vascular contractile force from typical experiments showing the effects of LP-NPY and αβ-m ATP on the vasoconstrictor responses to nerve stimulation in the presence of 0.1 μM prazosin. As shown in Fig. 6, the application of prazosin did not modify the first phase response at a low frequency (1 Hz), but slightly inhibited the first one at 4 Hz. At a high frequency (10 Hz), after treatment with prazosin (0.1 μM), the first phase response was inhibited by 50% (Fig. 6). The second phase vasoconstrictions were almost completely inhibited by prazosin at lower frequencies (1 and 4 Hz), and mostly inhibited at a high frequency (10 Hz), as previously reported (18). The remaining vasoconstrictions after 0.1 μM prazosin were not influenced by subsequent administration of 30 nM LP-NPY (Fig. 6). The remaining responses after 0.1 μM prazosin and 30 nM LP-NPY were completely suppressed by a subsequent application of 1 μM αβ-m ATP at any frequencies used, as shown in Fig. 6.

Effects of LP-NPY on the vasoconstrictor responses to administered NA and ATP

Intraluminally administered NA (0.003–1 nmol) and ATP (0.003–1 μmol) induced a dose-dependent vasoconstriction in the isolated, perfused canine splenic arteries. The treatment with LP-NPY (30 nM) slightly potentiated the vasoconstrictor responses to NA and ATP at any concentrations used, but not significantly (Fig. 7). The LP-NPY-induced potentiations were inhibited by a subsequent application of 1 μM BIBP 3226 (Fig. 7).

DISCUSSION

It has been considered that NPY Y1 receptor mechanisms are not of major importance for regulation of transmitter secretion (1), since the selective NPY Y1 receptor antagonists BIBP 3226 and SR120107A did not influence the prejunctional effect of NPY on the peripheral autonomic nervous system in rat vas deferens (13) or change nerve-stimulation-evoked NA or NPY overflow from pig kidney and spleen (10, 26). Thus, even in this study, it seems that NPY Y1 receptors mostly exist postjunctionally in peripheral vascular beds, although it was reported that LP-NPY increases the release of NA by KCl in the central nervous system (27). LP-NPY has been shown to potentiate the adrenergic contractile responses to nerve stimulation in the rat mesenteric vascular bed (8) and the epididymis of guinea pig (12). The present results confirmed that LP-NPY consistently potentiated the neuronal double-peaked vasoconstrictions in the isolated canine splenic artery. The results showed that the potentiation of LP-NPY of biphasic vasoconstrictions was completely inhibited by the blockade of α1-adrenoceptors or NPY Y1 receptors with prazosin or BIBP 3226, respectively. Furthermore, additional LP-NPY failed to potentiate the neuronal vasoconstrictions in the presence of prazosin. Thus, it is reasonable to consider that an activation of NPY Y1 receptors with LP-
NPY may selectively modulate the adrenergic transmission rather than the purinergic transmission of sympathetic cotransmission in the canine periartrial splenic nerve.

An α-adrenoceptor antagonist has been found to antagonize various effects of NPY (28, 29). It is also reported that prazosin blocked the NPY-induced vascular response of rat mesenteric arterial bed in the presence of NA, suggesting that prazosin is able to modify postjunctional NPY Y1 receptor activity in the presence of α1-adrenoceptor activation (30). It has been reported that NPY potentiates the vasoconstrictor response to nerve stimulation or exogenous administration of ATP and NA via activation of postjunctional NPY Y1 receptors (5–8). The present results demonstrated that LP-NPY potentiated the neuronal vasoconstrictor responses in the absence of α1-adrenoceptor blockade, but did not enhance the responses in the presence of α1-adrenoceptor blockade, showing that blockade of α1-adrenoceptors affects the postjunctional potentiation of LP-NPY. However, in the rabbit ear artery, NPY has been shown cause a postjunctional potentiation of neuronal vasoconstrictions in the presence or absence of prazosin (4). Moreover, Oberhauser et al. (5) also reported that NPY can enhance phenolamine-resistant renovascular depressive responses to nerve stimulation in the rat isolated, perfused kidney. Thus, it seems that the blockade of α-adrenoceptors may have no influence on the postjunctional synergistic effects of NPY in different species and/or organs. There is some evidence that multiple NPY receptor subtypes may be present in the postjunctional vascular effector (31–33). Thus, the possibility that NPY potentiates the α-blockade resistant, postjunctional purinergic contractile response via activation of LP-NPY-insensitive NPY receptors could not be excluded.

Several lines of evidence indicated that LP-NPY induces a distinct potentiation of vascular depressive responses induced either by nerve stimulation or by exogenous ATP and NA (6–8). However, in the present study, LP-NPY slightly but in parallel potentiated the exogenous ATP- or NA-induced vasoconstrictions, although it was not significant. On the other hand, LP-NPY markedly potentiated the neuronal adrenergic contractile response, whereas it failed to modify the neuronal purinergic contractile response in the present study. Studies on the postjunctional effects of NPY have implied that the capacity of NPY to potentiate the exogenous agonists-induced responses is nonspecific (34–36) and has characteristics of tissue- and
Fig. 6. Effects of LP-NPY and αβ-m ATP on the 0.1 μM prazosin-resistant first-peak (A) and the second-peak (B) vasoconstrictor response to electrical stimulation (ES; 10-V amplitude, 1-ms pulse duration and 30-s trains of pulses at frequencies of 1, 4 or 10 Hz). □: control, □: 0.1 μM prazosin, ■: 0.1 μM prazosin + 30 nM LP-NPY, ■: 0.1 μM prazosin + 30 nM LP-NPY + 1 μM αβ-m ATP. Data are presented as the mean ± S.E.M., n = 8. **P<0.01, as compared with the control group. *P<0.05, as compared with the preceding treatment.

Fig. 7. Effects of LP-NPY and BIBP 3226 on the vasoconstrictor response to exogenous NA (A) and ATP (B). □: control, □: 30 nM LP-NPY, ■: 30 nM LP-NPY + 1 μM BIBP 3226. Data are presented as the mean ± S.E.M., n = 6.

stimulation-induced adrenergic response readily caused a potentiation by NPY Y₁ receptor activation, but the injected NA-induced response did not. Since it was reported that junctional adrenergic receptors are not usual adrenoceptors in the rat basilar artery (38, 39), the possibility of differences between junctional and extrajunctional adrenoceptor subtypes cannot be ruled out. Moreover, we will investigate the interaction between junctional adrenergic α-receptors and NPY Y₁ receptor activation in the future.

Fried et al. (40) demonstrated that NPY is stored in NA in large dense-cored vesicles in unmyelinated axons of bovine splenic nerves. In addition, it has been also reported that reserpine interferes with the granular storage of monoamines, selectively causing the depletion of NA and NPY from sympathetic nerve terminals (41, 42), and no
depletion of ATP (43, 44). As suggested by Burnstock (45), ATP and NA are possibly released from two separate populations of exocytotic vesicles within the sympathetic nerve terminals. This idea is also supported by evidence obtained with canine splenic artery, where prolonged cold storage, which causes irreversible degeneration of adrenergic nerve fibers, may preferentially depress the co-transmission of the purinergic component, whereas the adrenergic component is largely unaffected (22, 23). Further observations showed that a smaller dose of tetrodotoxin, a selective sodium channel blocker inhibited only an adrenergic but not a purinergic component of double-peak vasconstrictor responses to nerve stimulation (20). Moreover, the blockade of adrenergic neuron with guanethidine preferentially inhibited the second-peak adrenergic but not the first-peak purinergic vasconstrictor responses (21). The present results that an activation of NPY Y1 receptors with LP-NPY selectively potentiated nerve-stimulated adrenergic rather than purinergic vasconstrictions may indicate that the functional synergism between NPY and NA is of physiological significance for costoring NA and NPY in sympathetic nerve terminals.

It has been suggested that endogenous NPY released from sympathetic nerves in the pig spleen mainly activates postjunctional NPY Y1 receptors, whereas circulating NPY preferentially influences prejunctional NPY Y2 receptors (10, 26). The evidence obtained with the canine splenic artery also confirms that exogenous NPY predominantly exerts a prejunctionally inhibitory modulation of the sympathetic transmission of the double-peak vasconstrictions induced by nerve stimulation (46). In addition, the blockade of NPY Y1 receptors with BBIBP 3226 has been shown to inhibit the slow, long-lasting sympathetic vasconstriction in guinea-pig vena cava (47). In the present study, treatment with BBIBP 3226 antagonizes the LP-NPY-induced potentiation and moreover the vasconstrictor responses are additionally inhibited. At low frequencies (1 and 4 Hz), BBIBP 3226 produced a significant inhibition of the second peak in the presence of LP-NPY, but did not affect the first peak. At a high frequency (10 Hz), BBIBP 3226 induced a slight, but significant inhibition of both the first- and second-peak responses. Thus, it is proposed that neuronal NPY via an activation of Y1 receptors may partially participate into the vasconstrictor responses to periarterial nerve stimulation in the canine splenic artery.

In conclusion, the results showed that there is a separate postjunctional potentiation of an activation of NPY Y1 receptors on adrenergic and purinergic components in sympathetic co-transmission. The synergism between activation of NPY Y1 receptors and nerve-stimulated postjunctional adrenergic responses may represent a functional characteristic of costoring NA and NPY in the sympathetic nerve terminals.

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