Functional Antagonism between Endogenous Neuropeptide Y and Calcitonin Gene-Related Peptide in Mesenteric Resistance Arteries

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ABSTRACT
To test the hypothesis that endogenous neuropeptide Y (NPY) counteracts the vasodilator effects of calcitonin gene-related peptide (CGRP), we used isolated mesenteric resistance arteries of rats and mice. With immunohistochemistry, we observed CGRP-containing fibers along and in the vicinity of a subset of NPY- or tyrosine hydroxylase-immunoreactive fibers. The CGRP1 receptor component calcitonin-related-like receptor was expressed by periarterial nerves and smooth muscle cells, whereas receptor activity-modifying protein 1 was observed primarily on the smooth muscle. In organ chambers, exogenous CGRP caused relaxations that were reversed by exogenous NPY. The effects were inhibited by 1-piperidinecarboxamide, \( N^2\)-[6-amino-1-[4-[4-pyridinyl]-1-piperazinyl]carbonyl)pentyl]amino]-1-[3,5-dibromo-4-hydroxyphenyl]-methyl]-2-oxoethyl]-4-(1,4-dihydro-2-oxo-3(2H)-quinazolinyl] (BIBN4096BS, a CGRP1 receptor antagonist; \( \text{pK}_\text{B} = 8.54 \pm 0.52 \)) and \((R)\)-NZ-(diphenylacetyl)-N-[4-hydroxyphenyl]methyl]argininamide (BIBP3226, a Y1 antagonist; \( \text{pK}_\text{B} = 7.00 \pm 0.49 \)), respectively. Pretreatment with capsaicin (1 \( \mu \text{M} \); 20 min) and the presence of BIBN4096BS (20 nM) increased contractile responses to \( \text{K}^+ \) (20–40 mM) and electrical field stimulation (EFS; 1–32 Hz). NPY increased contractile responses to \( \text{K}^+ \) and BIBP3226 (400 nM) reduced contractile responses to EFS. These effects were inhibited by capsaicin and BIBN4096BS, respectively. Furthermore, the relaxing effect of exogenous CGRP (10 nM) during phenylephrine-induced contraction (30 \( \mu \text{M} \)) was reversed by EFS, and this effect was reduced in the presence of BIBP3226. We confirmed that bioactive concentrations of endogenous CGRP and NPY can be released from periarterial sensory-motor and sympathetic nerves, respectively, and we demonstrate for the first time functional antagonism between endogenous NPY and CGRP at the level of the smooth muscle.

The potent 37-amino acid vasodilator calcitonin-gene related peptide (CGRP) can be released by perivascular sensory-motor nerves (for review, see Brain and Grant, 2004). CGRP contributes to cardiovascular homeostasis in the fetus (Thakor and Lavigne, 2005). This suggests that depending on their sensitivity (Yallampalli et al., 2002). It lowers blood pressure and vagal tone (Lavigne et al., 2005) and to the cardiovascular adaptations to pregnancy (Yallamplali et al., 2002). It lowers blood pressure and reduces end-organ damage in several experimental models (Deng and Li, 2005; Supowit et al., 2005; Márquez-Rodas et al., 2006). In experimental hypertension, changes in the expression and effects of CGRP either contribute to the increase in blood pressure or they play a compensatory role (Wang and Wang, 2004; Deng and Li, 2005; Supowit et al., 2005; Márquez-Rodas et al., 2006). Interventions that increase the efficacy of the vasodilator effects of endogenous CGRP could thus be a welcome addition to antihypertensive therapy. Enzymatic breakdown of CGRP (Fernandez-Patron et al., 2000), the density of CGRP-containing nerve fibers (Kawasaki et al., 1999), and the expression of CGRP receptor components (Zhang et al., 2006) are subjects of ongoing research. We hypothesize that effects of CGRP are modulated by other neuropeptides.

During development, afferent sensory-motor nerves and efferent postganglionic sympathetic nerves align with outgrowing arteries (for review, see Carmeliet and Tessier-Lavigne, 2005). This suggests that depending on their sensitivity...
tivity to neurotrophic, neuroattractant, and neurorepellent factors (Glebova and Ginty, 2005), the sensory-motor fibers might align with the sympathetic fibers within the arterial wall. The sympathetic neurotransmitters norepinephrine, ATP, and neuropeptide Y (NPY) are best known for their vasoconstrictor effects (e.g., Burnstock, 2004). These can be reduced by the relaxing effects of endogenous CGRP (Brain and Grant, 2004). Furthermore, NPY can inhibit through Y1 receptors the activity of the smooth muscle adenyl cyclase (Michel, 1998) that contributes to CGRP-induced vasodilatation (Brain and Grant, 2004). In addition, sympathetic neurotransmitters may modulate the release of CGRP through presynaptic effects on the sensory-motor nerves (Kawasaki et al., 1991).

In this study, we tested the hypothesis that endogenous NPY reduces CGRP-induced vasodilatation. In densely innervated mesenteric resistance arteries, we used immunohistochemistry to document the structural basis for interaction between sensory-motor and sympathetic nerves. In organ chamber studies, we recorded vasomotor responses of isolated resistance arteries to nonselective stimuli [K+-induced depolarization and electrical field stimulation (EFS)], recombinant neuropeptides, and selective ligands for TRPV1, CGRP1, and Y1 receptors (Doods et al., 1996, 2000; Caterina et al., 1997). Our results suggest that antagonists of Y1 receptors may be considered to enhance the arterial vasodilator effects of endogenous CGRP in vivo.

Materials and Methods
Experimental protocols were performed in accordance with institutional guidelines, and they were approved by the Ethics Committee on Experimental Animal Welfare of the Universiteit Maastricht.

Tissue Preparation. Twelve to 16-week-old male Wistar rats and C57BL/6J mice (Charles River, Maastricht, The Netherlands) were euthanized by CO2 inhalation. The small intestine and mesentery, thoracic aorta, femoral artery, and saphenous artery were isolated and harvested in aerated Krebs-Ringer bicarbonate (KRB) solution at room temperature. From the mesentery, first order arteries, second order (mouse) or third order (rat) side branches of the superior mesenteric artery were isolated; they were separated from venous, lymphatic, and periadventitial fat tissue; and then they were harvested in KRB. The endothelium was mechanically removed from part of the rat mesenteric small arteries (n = 6).

Histology. A 30-mm-segment of the small intestine and part of the arteries were fixed by incubation in 4% neutral buffered formalin (24 h; room temperature) and stored in 70% ethanol. Gut tissue was embedded in paraffin, and longitudinal sections (4 µm) were prepared. Vascular preparations were used as “whole mounts” (Stassen et al., 1997). On the paraffin sections, we used a peroxidase second step approach (swine anti-rabbit horseradish peroxidase). On the arterial whole mounts, we made use of fluorescent secondary antibodies (Alexa Fluor 488- or Alexa Fluor 546-labeled donkey anti-sheep or goat anti-rabbit IgG; Molecular Probes, Leiden, The Netherlands) and two-photon laser scanning microscopy (TPLSM) as described recently (Megens et al., 2007) with a 2100 multiphoton system (Bio-Rad, Hemel Hempstead, UK), a Tsunami Ti:SaSapphire laser (Spectra Physics, San Jose, CA), and an upright E400FN fluorescence microscope (Nikon, Tokyo, Japan). The primary antibodies were directed against rat αCGRP (1720-9004 and CA1137; BioTrend, Köln, Germany), NPY (RP1702; Amersham, St. Gilles Chalfont, UK), tyrosine hydroxylase (TH; 1017 381; Boehringer Mannheim, Mannheim, Germany), or the CGRP receptor components calcitonin-related-like receptor (CRLR) and receptor activity-modifying protein (RAMP) 1 that were gifts from Dr. C. Yalamalli (University of Galveston, Galveston, TX; Chauhan et al., 2004). For the TPLSM analyses, we made stacks of seven x,y images (750 nm in thickness each) abuminally from the autofluorescent external elastic lamina.

Vasomotor Responses. Two-millimeter segments of rat and mouse mesenteric resistance arteries were mounted between two stainless steel wires (40 µm in thickness) connected to a displacement device and an isometric force transducer (DSC6; Kistler Morse, Seattle, WA), respectively, in organ chambers (DMT, Aarhus, Denmark) filled with KRB solution at 37°C, and they were aerated with 95% O2, 5% CO2. The segments were progressively stretched to the diameter at which their contractile response to 10 µM norepinephrine was maximal. This optimal lumen diameter averaged 296 ± 13 µm (n = 45) and 214 ± 11 µm (n = 12) in rat and mouse mesenteric resistance arteries, respectively. We used two platinum electrodes placed along the long axis of the vessel segments and a stimulator (DMT) at supramaximal intensity and pulse duration (85 mA; 2 ms) to stimulate tetrodotoxin-sensitive neurogenic vasomotor responses (1–32-Hz EFS). Vessels were stimulated by EFS, the α1-adrenoceptor agonist phenylephrine (0.1–20 µM), or by increases in the extra-cellular potassium concentration (K+; 5.9–50 mM). They were relaxed by administration of human oCGRP (0.1–100 nM) or forskolin (0.1–10 µM), a diterpene that directly activates adenylyl cyclase. Exogenous recombinant NPY (0.1–100 nM) and the selective Y2 and Y5 agonist FPY3-38 (Michel, 1998) were used to reverse the relaxations. Experiments were repeated after persistent desensitization of sensory-motor nerves with capsaicin (1 µM during 20 min; Caterina et al., 1997; Szallasi and Blumberg, 1999), and/or in the presence of the nonselective and selective TRPV1 antagonists ruthenium red and capsazepine (Szallasi and Blumberg, 1999) and of the putative CGRP1 and Y1 receptor antagonists CGRP8-37, BIBN4096BS (Doods et al., 2000), and BIBP3226 (Doods et al., 1996), respectively.

Solutions and Drugs. The KRB solution contained 118.5 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 25.0 mM NaHCO3, and 5.5 mM glucose. High K+-KRB solution was in which all NaCl was replaced by KCl; solutions containing 5.9 to 50 mM K+ were prepared by mixing appropriate volumes of KRB and K+-KRB. All chemicals were obtained from Sigma-Aldrich (Geel, Belgium), and they were dissolved in bidistilled H2O, except for capsaicin, forskolin, and capsazepine (dissolved in ethanol) and BIBN4096BS (Doods et al., 2000) and BIBP3226 (Doods et al., 1996) (obtained from Dr. H. Doods, Boehringer Ingelheim Pharma KG, Biberach, Germany), which were dissolved in DMSO. At the concentrations used, the ethanol and DMSO solvents (<0.1%) did not elicit statistically significant effects.

Statistics. All structural and functional experiments were repeated in tissues from at least six animals. Functional observations were expressed as percentage of the maximal response to norepinephrine before experimental treatments. Sensitivity for agonists (pD2 = −log EC50; concentration of the agonist that produced 50% of the maximal response) was calculated by nonlinear regression curve fitting of individual agonist concentration-response curves (GraphPad Prism 2.0; GraphPad Software Inc., San Diego, CA). The apparent affinity for antagonists (pKb) was calculated using the Gaddum equation: pKb = log(CR − 1) − log[B], where CR is the ratio of the agonist EC50 values in the presence and absence of the antagonist, and [B] is the molar concentration of the antagonist. Analyses of variance with corrections for multiple comparisons (Bonferroni method) were applied to evaluate the statistical significance of effects. A P value <0.05 was used as criterion for statistical significance. Findings are reported as means ± S.E.M.

Results
Histological Observations
Figure 1 illustrates the distribution of neuropeptides and CGRP receptor components in the small intestine of the rat. CGRP is found in the submucosal and myenteric nerve
plexus and occasionally near the wall of arterioles. NPY is found in the submucosal plexus and abundantly around arterioles. Staining for CRLR is intense in intestinal and arterial smooth muscle. RAMP1, although seemingly less expressed in intestinal smooth muscle, is also clearly present in the arterial smooth muscle.

To demonstrate the vicinity of sensory-motor and sympathetic nerves, we used fixed whole preparations of isolated arteries that were labeled for CGRP and NPY. With TPLSM, we were able to image through the complete wall of the arteries, simultaneously exciting the specific fluorescent probes. Figure 2 illustrates neuropeptides and CGRP receptor components at the media-adventitia border of mesenteric resistance arteries of the mouse. CGRP is concentrated in fibers that are less dense than the NPY-immunoreactive fibers (Fig. 2, top). The former are situated somewhat more remotely from the arterial smooth muscle cells than the latter. It is noteworthy that the majority of the CGRP-positive fibers run on top of a subset of NPY-positive fibers. This is highlighted in the enclosed three-dimensional reconstruction (see Supplemental Movie). CRLR is clearly present on CGRP-containing nerves and on additional (presumably sympathetic) fibers (Fig. 2, middle). Although RAMP1 is expressed by cells in the vicinity of CGRP-positive fibers, it cannot be observed on perivascular nerve fibers (Fig. 2, bottom).

In mouse mesenteric resistance arteries, CGRP-containing fibers were also observed to be in the vicinity of TH-containing nerves (data not shown). Coalescence of CGRP-immunoreactive fibers with a subset of NPY- or TH-immunoreactive fibers was also observed in mesenteric resistance arteries of the rat (data not shown). Although the density of nerves differs considerably between types of artery, coalescence of CGRP- with NPY/TH-containing nerves was observed in all mouse arteries that we investigated so far, including beside mesenteric small arteries, the thoracic aorta, femoral artery, and saphenous artery (data not shown).

**Functional Observations**

**Effects of Agonists.** In isolated resting rat mesenteric resistance arteries, CGRP and NPY (0.1–100 nM) did not alter tone, whereas norepinephrine, phenylephrine, and increases in $K^+$ (5.9–50 mM) caused concentration-dependent contractions (Figs. 3–6). In arteries that had been made to contract, CGRP caused concentration-dependent and ultimately maximal relaxations (Fig. 4). The potency of the peptide was larger during contractions induced by $10^{-6}$ M phenylephrine (pD$_2$ of 9.7 ± 0.4) than during contraction induced by 40 mM $K^+$ (pD$_2$ = 7.8 ± 0.4), and it was intermediate during norepinephrine-induced contraction (pD$_2$ = 8.9 ± 0.5). In the latter case, the presence of the $\beta$-blocker propranolol (1 $\mu$M) significantly increased the vasodilator potency of CGRP (pD$_2$ = 9.5 ± 0.4).

In arteries that had been made to contract with 40 mM $K^+$, NPY caused concentration-dependent further increases in tone (Fig. 3). In arteries that had been made to contract with an agonist or with $K^+$ and subsequently to relax in response to 100 nM CGRP, NPY caused concentration-dependent (pD$_2$ = 7.8 ± 0.3), and ultimately, it caused complete reversal of the CGRP-induced relaxation (Fig. 4). NPY also reversed relaxing responses induced by the direct activator of adenylyl cyclase forskolin (1 $\mu$M; data not shown).

In contrast to NPY, the Y2 and Y5 receptor agonist PYY$_{3-36}$...
(0.01–1.0 μM) did not reverse CGRP-induced relaxation during agonist- or K\(^+\)-induced contraction (data not shown).

In addition, in mouse mesenteric resistance arteries that had been made to contract with K\(^+\), exogenous CGRP induced concentration-dependent relaxations that could be reversed by exogenous NPY. The sensitivity to the neuropeptide was comparable with that in rat mesenteric arteries.

**Effects of Capsaicin.** Ruthenium red (30 μM) and capsaicin (3 μM), a nonselective and a selective antagonist of TRPV1 channels (Szallasi and Blumberg, 1999), respectively, did not modify contractile responses to K\(^+\). The TRPV1-agonist capsaicin (1 μM), in contrast, initially reduced and then after 5 to 10 min resulted in an irreversible increase of the contractile responses to K\(^+\) (Figs. 3–5). Acute exposure to 1 μM capsaicin also markedly relaxed contractions induced by phenylephrine or norepinephrine. In contrast to K\(^+\), pretreatment with capsaicin did not significantly modify concentration-response curves to the contractile agonists (data not shown).

After treatment with capsaicin, NPY no longer enhanced contractile responses to K\(^+\) (Fig. 3), but CGRP still induced potent and marked relaxing responses (Fig. 4). Although pretreatment with the vanilloid resulted in a marked increase of the contractile response to 40 mM K\(^+\) (Figs. 3–5), the potency of CGRP to cause relaxation was not significantly modified (p\(_{D2}\) = 8.4 ± 0.4 versus p\(_{D2}\) = 7.8 ± 0.4; Fig. 4).

**Contributions of Substance P, Nitric-Oxide Synthase, and the Endothelium.** Substance P (0.1–100 nM) did not significantly alter contractile responses to 40 mM K\(^+\) in intact or capsaicin-treated rat mesenteric resistance arteries. Although mechanical removal of the endothelium and especially the presence of the nonspecific inhibitor of NO synthases N\(^\text{omega}\)-nitro-L-arginine methyl ester (0.1 mM) increased contractile responses to K\(^+\), they did not impair 1) acute relaxation in response to capsaicin (1 μM), 2) amplification of K\(^+\)-induced contraction after exposure to capsaicin (1 μM during 20 min), 3) relaxing responses to exogenous CGRP (0.1–100 nM), and 4) reversal of CGRP-induced relaxation (100 nM) by exogenous NPY (1–100 nM; data not shown).

**Effects of Antagonists.** Effects of antagonists of neuropeptide receptors were first evaluated in capsaicin-treated vessels made to contract with phenylephrine. CGRP\(_{8-37}\) (1 μM) reduced the relaxing responses to CGRP (0.1–100 nM) with low potency (p\(_{K_B}\) = 6.46 ± 0.52). The nonpeptidergic CGRP1 receptor antagonist BIBN4096BS (4, 20, and 100 nM) potently inhibited relaxing responses to CGRP (p\(_{K_B}\) = 8.54 ± 0.52). The nonpeptidergic Y1 antagonist BIBP3226 (0.4 μM) reduced the inhibitory effect of NPY on relaxing responses to CGRP and forskolin with similar potency (p\(_{K_B}\) = 7.00 ± 0.49).

To monitor release of endogenous neuropeptides, we next evaluated effects of the antagonists on the contractile responses to increasing concentrations of K\(^+\) in intact and capsaicin-treated arteries. BIBN4096BS (20 nM) significantly increased the contractile responses to low concentrations of K\(^+\) in intact but not in capsaicin-treated arteries (Fig. 5). BIBP3226 (0.4 μM) reduced the responses to high concentrations of K\(^+\) (>40 mM) in intact arteries and to low concentrations of K\(^+\) (20–40 mM) in desensitized arteries (Fig. 5). There were no statistically significant differences between observations in the presence of the CGRP antago-
To further explore release and interactions of endogenous neuropeptides, we evaluated effects of the antagonists on contractile responses to electrical field stimulation. The frequency-dependent contractions were moderately but significantly increased by BIBN4096BS (20 nM) and markedly reduced by BIBP3226 (0.4 μM) (Fig. 5). Adding CGRP receptor blockade to Y1 receptor blockade resulted in a partial restoration of contractile responses to electrical field stimulation (Fig. 5, bottom). The remaining neurogenic contractions could be completely blocked by the α1-adrenoceptor antagonist prazosin (1 μM) and by exposure to 6-hydroxydopamine (300 μg/ml during 10 min) (data not shown).

Both experiments demonstrate that vasodilator concentrations of endogenous CGRP and concentrations of endogenous NPY that enhance vasoconstriction can be released in the resistance arteries. To evaluate whether the latter involves inhibition of CGRP-induced relaxation we performed additional experiments (Fig. 6). Arteries were made to contract with a high concentration of phenylephrine (30 μM), and then they were relaxed by 10 nM exogenous CGRP and exposed to increasing frequencies of electrical field stimulation that reversed the relaxation induced by CGRP. Because the Y1 antagonist BIBP3226 (0.4 μM) reduced these neurogenic effects (Fig. 6), they can be attributed to endogenously released NPY.

**Discussion**

In small muscular resistance-sized arteries of rodents, CGRP-containing sensory-motor nerves run along NPY-containing sympathetic nerves, and the vasodilator effects of exogenous and endogenous CGRP are reversed by exogenous and endogenously released NPY.

We obtained qualitatively similar findings in rat and mouse arteries. Functional contractible and relaxing responses were more reproducible in rat vessels. Structural histological findings were clearer in the murine vessels as a result of the tighter organization of their walls.

The innervation of arteries comprises both efferent postganglionic sympathetic and primary afferent sensory-motor fibers, although their densities vary considerably between vascular beds and arterial branching orders (Bevan, 1983; Brain and Grant, 2004; Burnstock, 2004). Both types of innervation are particularly evident in the mesenteric arterial bed. In this bed, Luff et al. (2005) presented ultrastructural evidence for close apposition of varicosities of both types of nerves and for wider synaptic clefts in the case of sensory-motor compared with sympathetic nerves. We demonstrate
in this study that the CGRP-containing sensory-motor fibers run on top of a subset of NPY- or TH-containing fibers in small mesenteric arteries and in less densely innervated vessels such as thoracic aorta and femoral artery and in even more densely innervated arteries such as the saphenous artery. In view of developmental biological observations (Carmeliet and Tessier-Lavigne, 2005; Glebova and Ginty, 2005), this coalescence of CGRP-containing and sympathetic nerve fibers may be established during early stages of life. We conclude from our histological observations that there is a structural basis for interaction between the neurotransmitters of both types of nerves, and we hypothesize that this may involve postjunctional phenomena at the level of the effector smooth muscle cells.

Although substance P and neuronal nitric-oxide synthase are colocalized with CGRP in mesenteric periartrial sensory-motor nerves (Brain and Grant, 2004; Luff et al., 2005; Hatanaka et al., 2006), we found no evidence for their involvement in the functional responses that we monitored. However, we confirmed that CGRP is a potent vasodilator that can be released in vasodilator concentrations from the perivascular sensory-motor nerves (Brain and Grant, 2004; Hatanaka et al., 2006). The relaxing effect of CGRP was particularly clear during contractions induced by agonists such as phenylephrine and norepinephrine. This is relevant in view of the coalescence of CGRP-containing structures with sympathetic fibers. Our evidence for the release of vasodilator concentrations of endogenous CGRP comprises not only the previously published effects of TRPV1 agonist and electrical field stimulation on arterial vasoconstrictor responses (Caterina et al., 1991; Kawasaki et al., 1999; Yallampalli et al., 2002; Brain and Grant, 2004; Hatanaka et al., 2006) but also effects of comparatively small increases in extracellular potassium concentration. As judged from arterial responses before and after persistent sensory-motor nerve desensitization with capsaicin (Caterina et al., 1997; Szallasi and Blumberg, 1999), K⁺-induced contractions are accompanied by release of vasodilator components that 1) correspond to 5 to 10 nM exogenous CGRP and that 2) are sensitive to blockade by two CGRP1 receptor antagonists, the fragment CGRP₉₋₃₇ and the nonpeptidic blocker BIBN496BS (Dooods et al., 2000). K⁺-induced contractions were however not modified by ruthenium red and capsaicine, suggesting that K⁺-induced release of endogenous CGRP resulted from a depolarizing effect rather than from an action on the TRPV1 channels on sensory-motor nerves (Caterina et al., 1997; Szallasi and Blumberg, 1999).

CGRP receptors are heterotrimeric structures that couple to heterotrimeric G proteins. CRLR exhibits high affinity for CGRP in the presence of RAMP1 (Yallampalli et al., 2002; Chauhan et al., 2004; Zhang et al., 2006). We observed CRLR on sensory-motor nerves, sympathetic nerves, and arterial smooth muscle, whereas RAMP1 was found primarily on the smooth muscle. This confirms the earlier observations of Cottrell et al. (2005) except for the presence of RAMP1 in the vicinity but not on periarterial nerves. From this structural finding and the observed affinities for the antagonists CGRP₉₋₃₇ and BIBN496BS (Dooods et al., 2000), we conclude that the vasodilator effects of CGRP are mediated by postjunctional CGRP1 receptors. These receptors can stimulate release of endothelium-derived NO, K_ATP channels, and the activity of adenyl cyclase (for review, see Brain and Grant, 2004). In view of our observations in denuded arteries, in the presence of Nω-nitro-L-arginine methyl ester and during K⁺-induced contractions, cAMP production in arterial smooth muscle seems to play a major role in CGRP-induced relaxation. However, because the efficacy of CGRP was larger during agonist- than during K⁺-induced contractions, a contribution of K_ATP channels cannot be excluded. The difference between both types of contractions is, nevertheless, likely to result at least partly from release of endogenous CGRP during depolarization- but not agonist-induced contraction.

NPY that is stored in the vicinity of CGRP has been shown to enhance vasoconstrictor responses and to reduce vasodilator responses to stimulation of adenylyl cyclase (Dooods et al., 2000; Gradin et al., 2003). Our observations with NPY during K⁺-induced contraction and forskolin-induced relaxation are in line with these earlier findings. But, we also show for the first time that NPY can reverse relaxations induced by CGRP. In previous analyses of the interaction between NPY and CGRP using perfused vascular beds and bolus injections of neuropeptides (Kawasaki et al., 1991), this postjunctional functional antagonism could not be demonstrated, suggesting rather slow kinetics of the phenomenon. High-affinity antagonism by BIBP3226 and the lack of effect of PY3₅₋₃₆ indicate mediation by Y1 receptors that have been shown to inhibit adenylyl cyclase through pertussis toxin-sensitive G proteins (Aakerlund et al., 1990; Dooods et al., 2000).

As is the case for CGRP, bioactive concentrations of endogenous NPY can be released in mesenteric resistance arteries. However, Y1 antagonism resulted in more prominent inhibition of contractile responses to field stimulation than of those to K⁺-induced depolarization. Compared with the effects of CGRP1 receptor blockade and pretreatment with capsaicin, this suggests differences between sensory-motor and sympathetic nerves in terms of sensitivity for neurogenic stimuli. Field stimulation more readily triggers release of NPY, whereas K⁺-induced depolarization more readily stimulates release of CGRP. Release of different neurotransmitters by different stimuli has previously been documented, for example, for the cotransmitters norepinephrine, ATP, and NPY from the same sympathetic fibers (Burnstock, 2004). Release of endogenous CGRP by field stimulation, as revealed by the effect of CGRP1 receptor blockade on contractile responses to field stimulation, was particularly evident in the presence of the Y1 receptor antagonist. Both the previously observed prejunctional inhibitory effect of NPY on sensory-motor nerves (Kawasaki et al., 1991) and the postjunctional functional antagonism between neuropeptides that we observed, could be responsible for this observation.

Our last experiments intended to prove that sufficient endogenous NPY could be released to counteract the effects of CGRP. Our protocol was inspired by a previous study of Simonsen et al. (Gradin et al., 2003). We used a high concentration of phenylephrine to cause contraction and to saturate the postjunctional α₁-adrenoceptors that play a major role in neurogenic vasoconstriction. We next induced relaxation with exogenous CGRP, and we applied frequencies of electrical field stimulation that were shown before to release endogenous NPY. The stimulation caused reversal of the relaxing response to CGRP by a mechanism that was sensitive to Y1 blockade. This finding indicates that endogenously released NPY can counteract CGRP-induced relaxation. It also
suggests that even the receptors that are acted upon by exogenous CGRP are situated within the reach of the Y1 receptors stimulated by endogenous NPY.

Coalescence and functional antagonism of CGRP and NPY suggest that Y1 receptor blockade may be considered to enhance the efficacy of endogenous CGRP. In spontaneously hypertensive rats, which display an increased density of periarterial NPY-containing but not CGRP-containing nerves (Gradin et al., 2005; Dubinon et al., 2006), a role for endogenous CGRP herein has not been considered yet. Renal CGRP-containing sensory-motor nerves markedly affect renal function, and they reduce blood pressure even during stress-induced stimulation of the sympathetic nervous system (Zhao et al., 1997). In this model, BIBP3226 displays an age-dependent reducing effect on the enhanced neurogenic renovascular constriction (Y1 receptor antagonist: a review of its pharmacological properties. Regul Pept 19:355–360).

Fig. 7. Schematic overview illustrating coalescence of sensory-motor and sympathetic nerve fibers from which the neurotransmitters CGRP, NPY, and NE can be released by depolarization (K+)- and EFS. The α-adrenergic contraction of the smooth muscle (ellipsoid) can be reduced by the activity of adenylyl cyclase (AC), which is stimulated by CGRP1 receptors [composed of CRLR and RAMP1 (open box)], and it is inhibited by Y1 receptors.

References


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