Neuropeptide Y and ATP Interact to Control Renovascular Resistance in the Rat

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Abstract. Neuropeptide Y (NPY) and ATP are cotransmitters of norepinephrine (NE). Modulation of ATP-mediated purinergic neurotransmission by NPY was investigated in rat perfused kidney. β,γ-Methylene-L-ATP (β,γ-mATP; 1.0 to 1.5 μM, n = 8), NE (0.1 μM, n = 8), and NPY (0.1 μM, n = 14) increased perfusion pressure by maximally 12 ± 1, 17 ± 2, and 9 ± 1 mmHg, respectively. In the presence of NPY, responses to ATP and NE were dramatically enhanced. Renal nerve stimulation in the presence of the α-adrenoceptor antagonist phentolamine (1 μM) induced pressor responses of 54 ± 5 mmHg (n = 6). α-Blockade-resistant responses were abolished by the P2-purinoceptor blocker suramin (300 μM) and thus mediated by ATP. Purinergic responses were also reduced significantly (50%) by the NPY-Y1 receptor blocker BIBP 3226 (1 μM). NPY (0.1 μM) potentiated purinergic pressor responses and enhanced ATP release from 0.7 ± 0.2 to 4.1 ± 0.9 pmol (n = 4) associated with a significant increase of soluble ATPase activity. All NPY effects were prevented by BIBP 3226. Pressor responses to renal nerve stimulation delivered at short time intervals, mimicking enhanced sympathetic drive to the kidney, were not constant but showed a progressive rise, which was prevented by BIBP 3226. In this study, it is suggested that purinergic vasoconstriction in rat kidney depends on concomitantly released NPY. NPY by itself is only a weak vasoconstrictor but acts as a modulator of renal vascular resistance by enhancing the effects of its sympathetic cotransmitters, especially during sympathetic overactivity.

The sympathetic nervous system controls BP and renal function under physiologic and pathophysiologic conditions. Norepinephrine (NE) is believed to act as its major transmitter at postganglionic neuroeffector junctions through adrenoceptors, which can be divided in three classes: α₁-α₂, and β-receptors. In each class, at least three subtypes have been cloned (1). More recently, however, it has become clear that in addition to NE, other neurotransmitters such as neuropeptide Y (NPY) and ATP are released from sympathetic neurons (2,3) and may play an as yet underestimated role in situations of enhanced sympathoactive activity such as hypertension (4) and renal failure (5). NPY and ATP are both able to constrict renal blood vessels by activation of specific NPY (Y1)- (6) and ATP (P2X)-receptors (7), respectively. There is now substantial evidence from functional, radioligand binding and molecular genetic studies to support the view that such nonadrenergic vasoconstriction plays an important role in the kidney (8–11). We have shown in rat and human kidney that ATP is released from neuronal and non-neuronal sites (12,13) to increase renal vascular resistance. At low frequencies of renal nerve stimulation (RNS) or when α-adrenoceptors are completely blocked, pressor responses depend almost entirely on ATP (14–16). Our hypothesis is that the relative composition of all neurotransmitters released into the synaptic cleft—and not the quantity of an individual neurotransmitter—determines the extent of renal vasoconstriction. We claim that NPY modulates renovascular actions of its cotransmitters, especially ATP. Moreover, transmitter ATP inactivation is necessary to guarantee efficient control of synaptic transmission, and it has been suggested that a soluble ATPase released from sympathetic neurons together with ATP terminates the actions of ATP in the rat mesenteric vascular bed (17). In the present study, we tested whether NPY is an endogenous modulator of ATP release and purinergic pressor responses in rat kidney and whether NPY affects ATP degradation. BIBP 3226 was used as a selective nonpeptide Y1-receptor antagonist (18).

Materials and Methods
Male Wistar rats (Savo, Kisslegg, Germany) weighing between 250 and 350 g were used. Experiments were performed in accordance with institutional guidelines. Rats were anesthetized with sodium pentobarbital (60 mg/kg, intraperitoneally) and then the kidneys were isolated and perfused with Krebs-Henseleit solution according to a method described previously (19). Bipolar platinum electrodes were placed around the renal arteries to stimulate the renal nerves. Perfusion pressure was monitored continuously with a Statham P23 Db pressure transducer (Gould, Oxnard, CA) coupled to a Watanabe pen recorder (Graphitec Corp., Tokyo, Japan).

Experimental Protocol
The kidneys were perfused with drug-free modified Krebs-Henseleit solution at 37°C and a flow rate of 4.75 ml/min per g kidney wet weight. The kidney wet weights were calculated from previously published experiments, in which they were approximately 0.5% of the corresponding body weights (19). The perfusion solution was contin-
uously gassed with carbogen (5% CO₂/95% O₂) and passed through a 0.8-μm filter before it reached the kidneys. Immediately after preparation, a priming stimulation of 5 Hz for 30 s (1-millisecond width, 40 V) was delivered to test the viability of the preparation, which was followed by a stabilization period of 60 min. Drugs were either added to the perfusion solution after the priming stimulation (BIBP 3226, suramin, and phenotamine) or were infused into the perfusion line (β,γ-mATP, NE, and NPY) by a perfusion apparatus (Braun, Melsungen, Germany) at a constant flow rate of 0.158 mm/min. The perfusion of β,γ-mATP and NE into the perfusion line was stopped when the pressor responses to these agonists had reached a maximum. Details of the different experimental procedures are given in the Results section.

**Determination of Endogenous ATP and ATPase Activity**

The ATP content of 1-min fractions of the perfusate was measured with the luciferase technique, using the ATP bioluminescence FL-AAM assay kit (Sigma, Deisenhofen, Germany) and a Biolumat LB 953 luminometer (Berthold, Karlsruhe, Germany) as described previously (12) with a few alterations. One-minute samples of the effluent were collected on ice and analyzed within 30 min after collection. Blank values obtained with fresh Krebs–Henseleit solution were subtracted. The ATPase activity in the renal effluent, observed previously (17), was determined by measuring the degradation of exogenous ATP. One hundred microliters of 10 nM ATP were added to a 900-μl aliquot of each individual 1-min fraction and stored on ice (recovery sample). The amount of ATP present in each 1-min fraction of the perfusate was measured with the luciferase technique, using the ATP bioluminescence FL-AAM assay kit (Sigma, Deisenhofen, Germany) and a Biolumat LB 953 luminometer (Berthold, Karlsruhe, Germany) as described previously (12) with a few alterations. One-minute samples of the effluent were collected on ice and analyzed within 30 min after collection. Blank values obtained with fresh Krebs–Henseleit solution were subtracted. The ATPase activity in the renal effluent, observed previously (17), was determined by measuring the degradation of exogenous ATP. One hundred microliters of 10 nM ATP were added to a 900-μl aliquot of each individual 1-min fraction and stored on ice (recovery sample). The amount of ATP present in each 1-min fraction of the effluent and its individual recovery sample were determined from a standard calibration curve (0.001 to 5 nM ATP diluted in Krebs–Henseleit solution). The basal average ATPase activity for an individual kidney was estimated from the two 1-min recovery samples obtained at the start and the end of an experiment. The mean basal recovery was 81% obtained at the start and the end of an experiment. The mean basal recovery was 81 ± 3% (n = 8). All ATP values were corrected for recovery. Changes of ATPase activity in the 1-min fractions obtained during the course of an experiment were expressed as a percentage change from basal recovery. RNS-induced outflow of ATP was determined as the difference between this ATP content present in the three 1-min samples collected immediately after onset of stimulation and the estimated spontaneous ATP outflow (20).

**Drugs and Vehicles**

The Krebs–Henseleit solution had the following composition (mM): 118 NaCl, 4.7 KCl, 2.5 CaCl₂, 0.45 MgSO₄, 25 NaHCO₃, 1.03 KH₂PO₄, 11.10- (+)-glucose, 0.067 Na₂EDTA, and 0.07 ascorbic acid. A total of 35 g/L Hemaccel (polygelin) was added to the Krebs–Henseleit solution (21). The following drugs were purchased: rat neuropeptide Y (NPY) (Bachem, Heidelberg, Germany), (−)-norepinephrine (NE) HCl (Sigma, Deisenhofen, Germany), β,γ-methylene-γ-adenosine-5′-triphosphate tetrasodium salt (β,γ-mATP) (Biotrend, Research Biochemicals, Cologne, Germany), suramin hexosodium salt (Bayer, Wuppertal, Germany). Drugs were dissolved in distilled water before being diluted with Krebs–Henseleit solution for the NPY stock solution, NPY was dissolved in sodium phosphate buffer (0.1 M, pH 7.2).

**Statistical Analyses**

All data given are arithmetic means ± SEM. Differences between means were tested for significance by unpaired or paired t test modified according to Bonferroni. A probability level of P < 0.05 was considered significant.

**Results**

NPY Potentiates Pressor Responses to Exogenous NE and β,γ-mATP

The effect of NPY (0.1 μM) on NE and the stable ATP analogue β,γ-mATP (7)-induced pressor responses was tested in rat isolated kidneys. In each experiment, β,γ-mATP and NE were given in concentrations that induced comparable pressor responses (Figure 1). NE (0.1 μM; n = 8) induced pressor responses of 17 ± 2 mmHg (Figure 1B). An average of 1.2 ± 0.2 μM β,γ-mATP (n = 8) was necessary to induce pressor responses of 12 ± 1.0 mmHg (Figure 1B). Once the concentration of β,γ-mATP was adjusted for an individual kidney, it was kept constant throughout each experiment. Repeated addition of β,γ-mATP and NE in an alternating manner induced constant pressor responses (data not shown). NPY (0.1 μM) by itself increased renal perfusion pressure by only 9 ± 1 mmHg (n = 12) but markedly enhanced NE- and ATP-induced pressor responses (Figure 1, A and B). After cessation of NPY (0.1 μM) infusions, pressor responses returned to basal values within 10 min (data not shown).

**Figure 1.** Effect of neuropeptide Y (NPY) on pressor responses to exogenous β,γ-methylene-γ-ATP (β,γ-mATP) and norepinephrine (NE) in rat isolated kidney. Pressor responses to β,γ-mATP or NE were elicited in the absence and presence of NPY. Panel A shows a representative trace and Panel B gives mean data ± SEM of eight experiments. *P < 0.05, significant effect of NPY.
Adrenoceptor Blockade-Resistant Pressor Responses in Rat Kidney

In the presence of the nonselective α-adrenoceptor antagonist phentolamine (1 µM), pressor responses to RNS at 1 Hz were significantly increased (n = 6) (Figure 2). Pressor responses to NE (0.4 µM) were totally abolished by phentolamine (n = 5) (Figure 2). The α-adrenoceptor blockade-resistant pressor responses to 1 Hz were abolished by addition of the nonselective P2-purinoceptor antagonist suramin (300 µM) (Figure 3).

Effect of Exogenous NPY on Phentolamine-Resistant Pressor Responses at 1 Hz in Rat Kidney

When the renal nerves were stimulated twice (S2, S1; time interval, 6 min) in the presence of phentolamine (1 µM), both pressor responses were identical (Figure 4A, Table 1). NPY (0.1 µM), added before the second stimulation period (S2) in the presence of phentolamine, significantly potentiated these pressor responses (Figure 4B, Table 1). This NPY-induced potentiation of renal pressor responses was abolished by the selective NPY-Y1 receptor antagonist BIBP 3226 (1 µM) (Figure 4C, Table 1).

Role of Endogenous NPY in Phentolamine-Resistant Pressor Responses in Rat Kidney

To test whether endogenous NPY plays a role in purinergic pressor responses in rat kidney, BIBP 3226 (1 µM) was added to the perfusion solution before the second stimulation period (time interval between first and second stimulation, 6 min). In this first set of experiments, BIBP 3226 significantly decreased phentolamine-resistant pressor responses (n = 6) (Figure 4D, Table 1). Phentolamine-resistant pressor responses to RNS (1 Hz) delivered at short time intervals of 0.5 min were not constant but showed a progressive increase (n = 4) (Figure 5), in contrast to pressor responses delivered at a time interval of 6 min (Figure 4A). This progressive rise of pressor responses induced by repetitive RNS was prevented by BIBP 3226 (n = 4) (Figure 5).

Effects of NPY on RNS-Induced ATP Release and Soluble ATPase Activity in Rat Kidney

When the renal nerves were stimulated twice (S1, S2) in the presence of phentolamine (1 µM), there was a slight increase in ATP release into the perfusion solution of 0.7 ± 0.2 pmol.
NPY (0.1 μM), added before S2, increased RNS-induced ATP release approximately sixfold to 4.3 ± 1.0 (n = 4) (Figure 6A) and significantly enhanced ATPase activity (Figure 6A). These effects of NPY on RNS-induced ATP release and ATPase activity were prevented by addition of BIBP 3226 (1 μM) to the perfusion solution (Figure 6B). Boiling of the effluent for 5 min at 95°C or ultracentrifugation with a 30-kD membrane removed the ATPase activity (data not shown).

Discussion

The kidney is densely supplied with sympathetic nerve fibers, which represent the main communication link to the central nervous system. Only little evidence exists for a parasympathetic innervation (22,23). NE is the major renal sympathetic neurotransmitter and activates α₁- and α₂-adrenoceptors to induce renal vasoconstriction. Vasoconstriction-mediating α₂-adrenoceptors, however, play only a minor role for NE effects on vascular resistance in rat kidney (19). Besides NE, electrical stimulation of renal nerves in rat, pig, and human releases ATP (12,13) and NPY (24,25). RNS results in a frequency-dependent increase in perfusion pressure in rat isolated kidney (14–16). In general, it is believed that vasoconstrictory ATP effects are observed more easily at low, and vasoconstrictory NE effects at high, frequencies of stimulation. The aim of the present study was to test the hypothesis that endogenous NPY interacts with purinergic renal vasoconstriction. We used the rat isolated kidney preparation since we

Table 1. Pressor responses to renal nerve stimulation at 1 Hz (RNS) in rat isolated kidney in the presence of various drugs

<table>
<thead>
<tr>
<th>Drug</th>
<th>S1</th>
<th>S2</th>
</tr>
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<tbody>
<tr>
<td>Phentolamine (throughout)</td>
<td>51 ± 9</td>
<td>56 ± 7</td>
</tr>
<tr>
<td>Phentolamine (throughout), BIBP 3226</td>
<td>45 ± 8</td>
<td>21 ± 3</td>
</tr>
<tr>
<td>Phentolamine (throughout), NPY in S2</td>
<td>50 ± 5</td>
<td>144 ± 7</td>
</tr>
<tr>
<td>Phentolamine/BIBP 3226 (throughout)</td>
<td>21 ± 4</td>
<td>18 ± 3</td>
</tr>
</tbody>
</table>

* There were two periods (S1, S2) of RNS, elicited in 6- to 7-min intervals. Phentolamine (1 μM; Figure 4, A, B, and D) or a combination of phentolamine and BIBP 3226 (each 1 μM; Figure 4C) was present for both stimulation periods (throughout). NPY (0.1 μM; Figure 4, B and C) or BIBP 3226 (1 μM; Figure 4D) was added before S2. Data are means ± SEM of six to 10 experiments.

**Discussion**

The kidney is densely supplied with sympathetic nerve fibers, which represent the main communication link to the central nervous system. Only little evidence exists for a parasympathetic innervation (22,23). NE is the major renal sympathetic neurotransmitter and activates α₁- and α₂-adrenoceptors to induce renal vasoconstriction. Vasoconstriction-mediating α₂-adrenoceptors, however, play only a minor role for NE effects on vascular resistance in rat kidney (19). Besides NE, electrical stimulation of renal nerves in rat, pig, and human releases ATP (12,13) and NPY (24,25). RNS results in a frequency-dependent increase in perfusion pressure in rat isolated kidney (14–16). In general, it is believed that vasoconstrictory ATP effects are observed more easily at low, and vasoconstrictory NE effects at high, frequencies of stimulation. The aim of the present study was to test the hypothesis that endogenous NPY interacts with purinergic renal vasoconstriction. We used the rat isolated kidney preparation since we

(n = 4) above basal in S1 (Figure 6A). NPY (0.1 μM), added before S2, increased RNS-induced ATP release approximately sixfold to 4.3 ± 1.0 (n = 4) (Figure 6A) and significantly enhanced ATPase activity (Figure 6A). These effects of NPY on RNS-induced ATP release and ATPase activity were prevented by addition of BIBP 3226 (1 μM) to the perfusion solution (Figure 6B). Boiling of the effluent for 5 min at 95°C or ultracentrifugation with a 30-kD membrane removed the ATPase activity (data not shown).

Figure 4. Effect of NPY and the NPY-Y1 receptor-selective antagonist BIBP 3226 on α-adrenoceptor blockade-resistant pressor responses to RNS at 1 Hz in rat isolated kidney. Pressor responses were elicited in 6- to 7-min intervals. Phentolamine (1 μM) was given throughout the experiments. BIBP 3226 (1 μM) was added either throughout or before S2. NPY (0.1 μM) always was added 3 to 4 min before S2. (A) A representative trace of control experiment. (B) Effect of NPY on phentolamine-resistant pressor responses. (C) Effect of NPY on pressor responses to RNS in the presence of phentolamine and BIBP 3226. (D) Effect of BIBP 3226 on α-adrenoceptor blockade-resistant pressor responses. Mean data ± SEM of changes in perfusion pressure for six to 10 experiments are given in Table 1.

*P < 0.05, significant effect of NPY or BIBP 3226.
n knew from previous experiments that under certain conditions RNS-induced pressor responses are largely ATP-dependent (12,15,16), giving us the unique possibility to study our hypothesis.

NPY and Pressor Responses to Exogenous NE and ATP

NPY (0.1 μM) by itself only slightly increased basal perfusion pressure in isolated kidneys of Wistar rats. Our findings are consistent with reported small or even negligible effects of exogenous NPY to produce direct vasoconstriction in rat renal interlobar (26) and mesenteric arteries (27,28). However, Oellerich and Malik had observed a rather pronounced increase in perfusion pressure by NPY in kidneys of Sprague Dawley rats (24). The reason for this discrepancy is not known. Nevertheless, in the present study NPY dramatically potentiated the effect of exogenous NE. Similar effects of NPY have been shown previously for other sympathetically innervated tissues (2,16), including the kidney (24). The increase in perfusion pressure elicited by the stable P2X-purinoceptor agonist β,γ-mATP was potentiated by NPY to an extent comparable to that of NE. NPY modulation of P2X-purinoceptor-induced vasoconstriction is likely to be mediated by the NPY-Y1 receptors, since in the rat mesenteric bed the Y1-receptor-selective antagonist BIBP 3226 had blocked NPY-induced potentiation of pressor responses to ATP (27,28).

Effects of NPY on RNS-Induced Purinergic Neurotransmission

The nonselective α-adrenoceptor antagonist phentolamine abolished the effect of exogenous NE (0.4 μM) in rat kidney, indicating that all vasoconstriction-mediating α-adrenoceptors were blocked. However, RNS-induced pressor responses were not reduced by phentolamine but instead significantly increased. These α-adrenoceptor blockade-resistant pressor re-
sponses were blocked by the nonselective P2-purinoceptor antagonist suramin and thus reflect purinergic pressor responses due to neurally released ATP. RNS-induced pressor responses in the presence of phentolamine were greater than in its absence since blocking prejunctional inhibitory α₁-β-adrenoceptors enhances ATP release (12, 25). Exogenous NPY (0.1 μM) potentiated these renal purinergic pressor responses, and this effect was prevented by BIBP 3226, thus supporting the view of an involvement of Y1-receptors. NPY has been shown to inhibit NE release in human kidney by about 20% (29) and neuronal ATP release in guinea pig vas deferens (30). This effect of NPY is probably due to activation of prejunctional NPY receptors of the Y2-subtype (2), which are not blocked by BIBP 3226. In contrast to these observations, RNS-induced ATP release was enhanced by NPY in the present experiments. Therefore, the enhancement of ATP release was probably due to a postjunctional effect of NPY, since it was abolished by BIBP 3226. Non-neuronal release of extracellular ATP following RNS in rat-isolated kidney has been observed previously (12). Thus, it is more feasible that NPY potentiated a postjunctional ATP release process; whether this is a receptor-operated process or simply due to vasoconstriction is under investigation.

We then used BIBP 3226 to test whether endogenous NPY plays a role in renal nerve-mediated purinergic vasoconstriction. And, indeed, when the NPY-Y1 receptor antagonist BIBP 3226 was added to the perfusion solution, it inhibited these phentolamine-resistant ATP-mediated pressor responses by 50%. This was not a nonspecific effect of BIBP 3226, since it did not alter β,γ-mATP-induced increase of renal perfusion pressure (data not shown). To our knowledge, this is the first evidence for a role of endogenous NPY to modulate physiologic actions of ATP in the kidney and supports in vivo findings in reserpine-treated pigs, in which BIBP 3226 reduced renal vasoconstriction to sympathetic nerve stimulation by 60% (31). In the rat mesenteric vasculature, constrictor responses elicited by nerve stimulation were also inhibited by BIBP 3226 (28), whereas an increase in renal vascular resistance elicited by an α₁-β-adrenoceptor agonist methoxamine (32) was not influenced by BIBP 3226. These data show that endogenous NPY by itself is not able to produce renal vasoconstriction but potentiates vascular effects of its sympathetic cotransmitters, especially ATP.

There is some evidence to suggest that neurally released ATP is rapidly broken down by a concomitantly released, soluble ATPase in the rat mesenteric bed (17). Such a soluble ATPase activity was found also in the renal effluent of rat kidneys. The identification and characterization of the ATPase formed in rat kidney were not the primary aims of the present study. However, our findings may shed some light on previous experiments pursuing the idea of a purinergic neurotransmission and its modulation by prejunctional receptors in rat kidney and possibly other isolated tissues by simply measuring ATP release. The functional evidence favored a role of ATP as a fast neurotransmitter at lower stimulation frequencies (0.3 to 4 Hz) (14–16), whereas ATP release was reliably measurable only at higher frequencies (>4 Hz) (12). This obvious discrepancy may be explained by rapid breakdown of neurotransmitter ATP by neurally released ATPases. Moreover, one must keep in mind that drugs or endogenous substances such as NPY may modulate ATPase activity. It is therefore advisable to assess drug effects on ATPase activity to prevent misinterpretations.

It is known that ATP and NPY have different time spans of action. ATP acts as a fast neurotransmitter via opening of ion channels in milliseconds, whereas NPY has a more long-lasting effect via Ca²⁺ mobilization (2). One can therefore imagine that NPY plays a role in clinical conditions of sympathetic overactivity such as hypertension and heart and renal failure. Accordingly, plasma levels of NPY were increased in patients with hypertension (4) and chronic renal failure (5). When we mimicked a situation of enhanced sympathetic drive to the kidney by delivering a train of three consecutive periods of 30 pulses, pressor responses to RNS were not stable but increased from one stimulation period to the next. This increment in RNS-induced pressor responses was due to endogenous NPY since it was totally prevented by BIBP 3226.

In summary, NPY by itself appears to be only a weak renal vasoconstrictor. NE and ATP-mediated renal vasoconstriction is markedly potentiated by NPY in rat kidney. Endogenous NPY acts as a modulator of vascular effects of its cotransmitters ATP and NE. This NPY involvement takes place already at physiologic rates of renal nerve firing. Even more important, our data may be interpreted as evidence for a role of endogenous NPY potentiating vascular effects of ATP in situations of sympathetic overactivity.

Acknowledgments

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