Neuropeptide Y inhibits potassium-stimulated glutamate release through Y₂ receptors in rat hippocampal slices *in vitro*

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1 We investigated the effects of neuropeptide Y (NPY), peptide YY (PYY), NPY_{13–36}, NPY_{18–36}, [Leu³¹][Pro³⁴]NPY and of pancreatic polypeptide Y (PPY) on calcium-dependent, potassium-stimulated glutamate release in superfused rat hippocampal slices.

2 NPY, PYY and the Y₂ receptor agonist NPY_{13–36} equipotently inhibited the release of glutamate. The half-maximal response was observed at about 10 nM in a dose-dependent manner (3 to 100 nM). Maximal inhibition of 50 to 60% was obtained at 100 nM. At higher concentrations of the peptides (300 nM and 1 µM) this inhibition was partially or entirely reversed. Porcine NPY_{13–36} and NPY_{18–36} inhibited glutamate release by about 44% at 100 nM.

3 The specific Y₁ receptor agonist, [Leu³¹][Pro³⁴]NPY, caused an insignificant increase in glutamate release at 100 to 300 nM concentrations. PPY had no effect on potassium-evoked glutamate release in hippocampal slices at concentrations of 30 nM to 1 µM.

4 The experiments support previous electrophysiological data. They suggest a potent inhibitory action of NPY through NPY-Y₂ receptors on the release of the excitatory amino acid glutamate in rat hippocampus. Especially under conditions of increased NPY synthesis, such as in epilepsy, this mechanism may be of pathophysiological relevance.

Keywords: Glutamate; hippocampus; neuropeptide Y; NPY-Y₂ receptor; presynaptic inhibition; hippocampal slice; epilepsy; neurotransmitter release

Introduction

Neuropeptide Y (NPY), a 36-amino acid peptide is a member of a family of peptides that also includes peptide YY (PYY) and pancreatic polypeptide Y (PPY) (Tatemoto, 1982; Tatemoto *et al.*, 1982). NPY is abundant in the central and peripheral nervous system (Allen *et al.*, 1983; Chronwall *et al.*, 1985; DeQuidt & Emson, 1986; Morris, 1989). Two receptor subtypes (Y₁ and Y₂) equally recognizing NPY and PYY have been defined by receptor binding studies (Aicher *et al.*, 1991; Dumont *et al.*, 1993). A specific ligand of the Y₁ receptor is the NPY derivative [Leu³¹][Pro³⁴]NPY (Fuhlen-dorff *et al.*, 1990). Selective agonists of the Y₂ receptor are amino-terminally truncated analogues of NPY such as NPY_{13–36} (Wahlestedt *et al.*, 1986). Both receptor subtypes are abundant in the central nervous system (Walker & Miller, 1988; Martel *et al.*, 1990; Gehlert *et al.*, 1992; Dumont *et al.*, 1993). The existence of a third receptor species (Y₃) with low affinity for PYY has been postulated recently (Grundemar *et al.*, 1991).

A variety of behavioural effects are observed after infusion of NPY into the central nervous system. Thus NPY stimulates food and water intake through Y₁ receptors (Stanley & Leibowitz, 1985; Danho *et al.*, 1988). It facilitates memory retention probably via Y₂ receptors (Flood & Morley, 1989). Activation of Y₁ or Y₂ receptors may affect locomotor behaviour in an inhibitory or stimulatory fashion, respectively (Heilig & Murison, 1987; Heilig *et al.*, 1988). Blockade of Y₁ receptor synthesis by infusion of an antisense oligonucleotide to Y₁ receptor mRNA into the amygdala has an anxiogenic effect (Wahlestedt *et al.*, 1993).

Induction of NPY expression has been observed in various animal models of epilepsy (Marksteiner & Sperk, 1988; Sten-fors *et al.*, 1989; Marksteiner *et al.*, 1989, 1990a; Bellmann *et al.*, 1991). Mossy fibres, which represent an important excitatory pathway of the hippocampal formation, exhibit a

marked and long lasting increase in NPY immunoreactivity (Marksteiner *et al.*, 1990b; Sperk *et al.*, 1992). It has been conjectured that NPY released during epileptic seizures may serve a protective role in inhibiting glutamate release from mossy fibre terminals (Marksteiner *et al.*, 1990b). Electro-physiological studies supported this proposal: NPY exerts a presynaptic inhibitory effect on the glutamatergic transmission at Schaffer collateral/CA1 synapses and at mossy fibre/CA3 synapses of the hippocampus (Colmers *et al.*, 1985; 1988; Haas *et al.*, 1987; Klapstein & Colmers, 1993). These effects seem to be mediated by presynaptic Y₂-receptors possibly by inhibiting Ca²⁺ influx through N-type Ca²⁺ channels (Colmers *et al.*, 1991; Toth *et al.*, 1993). Thus the aim of our present study was to investigate the effects of NPY and NPY receptor agonists on glutamate release in superfused slices of the rat hippocampus by means of biochemical methods.

Methods

Superfusion experiments

Adult male Sprague-Dawley rats (300–350 g; Versuchstier-zuchtanstalt, Himberg, Austria) were decapitated and their brains were rapidly removed into ice-cold Krebs-Henseleit-Tris buffer (composition mM: NaCl 118, KCl 4.8, MgSO₄ 1.3, CaCl₂ 1.2, NaHCO₃ 15, KH₂PO₄ 1.2, Tris 10, glucose 10, adjusted to pH 7.3 by gassing with 95% O₂/5% CO₂). Superfusion experiments were performed according to Burke & Nadler (1988). The hippocampi were removed and 300 µm thick transverse slices were obtained with a McIlwain tissue chopper. Two slices were transferred to a small circular nylon mesh net located in a superfusion chamber (Singer, 1988). They were covered by a second nylon mesh net and superfused at a flow rate of 1 ml min⁻¹ with the above buffer. The medium was continuously gassed with 95% O₂/5% CO₂. The system was kept at 34°C. Transmitter release was induced by increasing the K⁺ concentration (usually to 40 mM) for 90 s. The Na⁺ concentration was correspondingly reduced. The

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slices were stimulated twice (after about 50 and 100 min respectively).

The respective peptide was added to the superfusion medium 8 min before the second pulse of elevated K^+ . The system was changed to a previously well gassed buffer (50 ml contained in a siliconized glass flask). The respective NPY analogue (in 500 μ l 1.1% bovine serum albumin) was admixed immediately before use. In order to avoid extensive foaming and adsorption of the peptide to the glass flask, gassing was stopped. (This interruption of gassing for 9.5 min did not affect the basal or the K^+ stimulated glutamate release). The peptide was included in the same way in the medium containing elevated K^+ . After the stimulation period the perfusion system was changed back to the original continuously gassed superfusion medium. Four perfusion chambers were used in parallel. In the control experiments slices were superfused in the same way, including bovine serum albumin without peptide, prior to and during the second K^+ -stimulus. Fractions of 1 ml were collected and lyophilized. At the end of the experiment the brain slices were transferred to 1 ml 0.1 M $HClO_4$ and sonicated. The homogenate was centrifuged (15,000 g , 10 min). The supernatants were diluted 1:80 with 0.4 M $NaBO_4$ buffer, pH 10.0 for determination of tissue glutamate. Pellets were solubilized in 0.5 M $NaOH$ for protein determination (Lowry *et al.*, 1951). Recovery of NPY, NPY_{13-36} , NPY_{18-36} and PYY as monitored by radioimmunoassay (Bellmann *et al.*, 1991). The NPY analogues cross-reacted about 10 to 30% with our NPY antibody. The recovery of the peptides was generally more than 95%; values were therefore not corrected. The antibody did not significantly crossreact with $[Leu^{31}][Pro^{34}]$ NPY or PPY.

Determination of glutamate by h.p.l.c.

Amino acids were determined by high performance liquid chromatography (h.p.l.c.) and fluorimetric detection after pre-column derivatization with *o*-phthalaldehyde. The lyophilized samples were solubilized in 200 μ l 0.4 M $NaBO_4$ buffer, pH 10.0, containing 400 nM L-cysteic acid (Sigma) as internal standard. Aliquots (120 μ l) of the samples were placed into an automatic h.p.l.c. injection system (Hitachi-Merck) refrigerated at 6°C. Each sample was reacted with 20 μ l *o*-phthalaldehyde reagent immediately prior to injection. (The *o*-phthalaldehyde reagent was prepared daily by solubilizing 5 mg *o*-phthalaldehyde in 93 μ l methanol and adding of 833 μ l 0.4 M $NaBO_4$ buffer, pH 9.5 and 4 μ l β -mercaptoethanol. The mixture was incubated at room temperature overnight before use). The reaction was stopped after 90 s by addition of 80 μ l 1 M phosphate buffer pH 1.5 and mixing. An aliquot of 180 μ l was then injected into the h.p.l.c.

Separation of amino acids was performed with a C18 reversed phase column (Merck, LiChrospher 100, 5 μ m, 4 $\times$ 125 mm). The mobile phase consisted of a mixture of a 0.1 M NaH_2PO_4 buffer, pH 6.2 containing 20% methanol (eluent A) and a 0.01 M NaH_2PO_4 buffer, pH 5.8, 80% methanol (eluent B). The column was eluted isocratically for 7 min with 95% eluent A and 5% eluent B followed by a gradient increasing the concentration of eluent B to 50% during 3 min at a flow rate of 1 ml min^{-1} . After washing the column with eluent B for 5 min and equilibrating it with 95% eluent A/5% eluent B (5 min) the next sample was applied. The amino acids were monitored with a fluorescence detector (Shimadzu, RF 530) at 340 nm excitation and 455 emission. The glutamic acid derivative eluted after 8 min and was quantified with external standards (1 to 50 pmol) included after every sixth sample. The recovery was determined by the internal standard L-cysteic acid.

The amount of potassium-stimulated glutamate release was calculated from those two fractions obtained during stimulation with high K^+ . Basal release rates were estimated from the fractions prior to stimulation and were subtracted. The

ratio of glutamate released during the second and the first stimuli (S_2/S_1) was calculated. It was 1.02 ± 0.08 ($n = 32$) in control experiments.

Human NPY_{13-36} was custom synthesized by Neosystems (Strasbourg, France) and purified by h.p.l.c. All other neuropeptides were obtained from Peninsula (Great Britain).

Results

After a recovery period of about 30 min a basal release rate of glutamate of 4 to 7 pmol mg^{-1} protein min^{-1} was observed. Elevated K^+ concentrations caused a dose-dependent increase in glutamate release. At 40 mM KCl (90 s) 416 ± 32 (17) pmol glutamate mg^{-1} protein was released during a period of 2 min representing about 0.6% of total glutamate in the slices. This release was Ca^{2+} -dependent. Substituting Ca^{2+} in the superfusion medium by 2 mM EGTA reduced the K^+ -stimulated release by 90% (Figure 1b). Rates of basal and K^+ -stimulated release of aspartate were 34 ± 3.3 (12) and 107 ± 3.4 (7) pmol mg^{-1} protein min^{-1} , respectively. Markedly elevated aspartate release (accompanied by elevated glutamate release) was observed only occasionally and was taken as indicator for Ca^{2+} -independent release. These experiments were not included.

NPY at concentrations of 30 to 100 nM inhibited potassium-stimulated glutamate release by 45 to 55% (Figure 2). PYY and the Y_2 receptor agonist NPY_{13-36} were about as potent as NPY (Figure 2, Table 1). In Figure 2 dose-response

Figure 1 K^+ -stimulated glutamate release from hippocampal slices and its Ca^{2+} dependency. (a) Two hippocampal slices (300 μ m) were superfused. For stimulation K^+ concentration was increased to 50 mM for 90 s. Glutamate was determined in 1 ml (1 min) fractions by h.p.l.c. (b) Eight min prior to the second stimulus Ca^{2+} was substituted by 2 mM EGTA.

Figure 2 Dose-response curve for inhibition of K^+ -stimulated glutamate release by rat NPY ($\text{—}\diamond\text{—}$) and rat NPY_{13-36} ($\text{--}\square\text{--}$). S_2/S_1 values were calculated from K^+ -stimulated release in the presence and absence of the respective peptides.

relationships for NPY and NPY_{13-36} are shown. Maximal inhibition (56%) was observed at a concentration of 100 nM. Half maximal inhibition was obtained at about 10 nM. At concentrations of 300 nM to 1 μM , inhibition of K^+ -evoked glutamate release by NPY_{13-36} was less prominent (about 30%) or entirely reversed (NPY, PYY, Figure 2, Table 1).

In Table 1 the efficacies of NPY and of the NPY analogues in inhibiting glutamate release are compared. At concentrations of 100 nM, porcine NPY_{13-36} and NPY_{18-36} were slightly less effective (about 44% inhibition) than rat NPY, rat NPY_{13-36} and PYY (56% inhibition). PYY had no significant inhibitory action upon glutamate release. There was a slight increase in release of the amino acid in the presence of 100 nM $[\text{Leu}^{31}][\text{Pro}^{34}]\text{NPY}$.

Discussion

Our experiments show equipotent inhibitory actions of NPY, PYY and the Y_2 receptor agonist NPY_{13-36} on K^+ -stimulated glutamate release in hippocampal slices. The specific Y_1 receptor agonist $[\text{Leu}^{31}][\text{Pro}^{34}]\text{NPY}$ lacked this inhibitory action on glutamate release. The experiments are in good agreement with electrophysiological data by Colmers and coworkers (Colmers *et al.*, 1985; 1988; 1991; Klapstein & Colmers, 1993). The authors demonstrated an inhibitory action of NPY upon excitatory postsynaptic potentials and population spikes in CA1 and CA3 pyramidal neurones after orthodromic stimulation. This action of the peptide is mediated by presynaptic Y_2 receptors (Colmers *et al.*, 1988; 1991).

The present experiments demonstrate this effect of NPY and the Y_2 receptor agonist NPY_{13-36} at low concentrations (half maximal inhibition at about 10 nM) in superfused slices of the rat hippocampus. Maximal inhibition of glutamate

release occurred at 100 nM NPY and was about 55%. This suggests that not all glutamate terminals may be under inhibitory control of the peptide. This is in agreement with Colmer's results and the observation that NPY for example was inactive at perforant path-granule cell synapses (Klapstein & Colmers, 1993).

It is also interesting that maximal inhibition of glutamate release observed at 100 nM was attenuated at higher concentrations of NPY, PYY and NPY_{13-36} (300 nM and 1 μM). The latter effect could be mediated through a different type of NPY receptor. Monet *et al.* (1992) reported an agonist action of NPY with glutamate at the N-methyl-D-aspartate receptor at high nM concentrations. Stimulation of these receptors would result in enhanced release of glutamate. It also may be possible that NPY_{13-36} exerts a partially antagonistic action at the higher concentrations. Such an antagonistic action has been reported for NPY_{18-36} on the inhibitory action of NPY upon isoprenaline-stimulated adenylyl cyclase in cardiac ventricular membranes (Balasubramaniam & Sheriff, 1990). The low concentrations of NPY required for inhibition of glutamate release are in favour of a physiological role for this mechanism.

The sites for this action of NPY on glutamate release within the hippocampal formation may be at CA1 pyramidal neurones innervated by Schaffer's collaterals or at mossy fibre-CA3, commissural-CA3 and CA3-CA3 synapses (Klapstein & Colmers, 1993). The cellular origin of NPY exerting this action is not entirely clear. NPY is contained at high concentrations in interneurons throughout the hippocampus (Köhler *et al.*, 1986). A recent electron microscopy study indicates the presence of NPY immunoreactive varicosities in immediate apposition to glutamatergic presynaptic terminals (Milner & Veznedaroglu, 1992). NPY originating from these neurones may control glutamate release under physiological conditions.

Another compartment that may release NPY is the glutamatergic nerve terminal itself. No NPY, however, has been found in granule cells or in pyramidal neurones of untreated rats (Köhler *et al.*, 1986; Morris, 1989). In epileptic rats, however, a markedly augmented and long lasting expression of NPY has been observed in granule cells of the dentate gyrus and, transiently, also in CA1 and CA3 pyramidal neurones (Marksteiner *et al.*, 1990b; Sperk *et al.*, 1992). Pre-existing Y_2 receptors may serve as targets for NPY released together with glutamate from these neurones. The peptide is released at a high rate during epileptic seizures. It then may exert its action by suppressing the release of the excitatory neurotransmitter and inhibiting seizure propagation.

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Table 1 The effects of neuropeptide Y (NPY) and NPY analogues on K^+ -stimulated glutamate release

	30 nM	% inhibition at 100 nM	300 nM
NPY_{1-36} (rat)	47 ± 8.7 (3)*	56 ± 2.0 (3)*	-0.2 ± 5.3 (7)
PYY (rat)	ND	52 ± 9.8 (4)*	-17 (2)
NPY_{13-36} (rat)	44 ± 3.6 (5)**	56 ± 5.1 (6)**	27 ± 1.5 (3)
NPY_{13-36} (porcine)	ND	44 ± 2.1 (3)*	ND
NPY_{18-36} (porcine)	ND	44 (2)	ND
$[\text{Leu}^{31}][\text{Pro}^{34}]\text{NPY}$ (porcine)	11 ± 0.4 (3)	-18 ± 1.4 (5)	-0.1 ± 15.3 (5)
PPY	ND	7.3 ± 7.0 (3)	ND

* $P < 0.005$, ** $P < 0.001$ significantly different vs controls; * $P < 0.05$ significantly different vs 30 nM (Student's *t* test); ND, not determined. Data represent mean reduction in S_2/S_1 values expressed as % of control values $\pm$ s.e.mean.
(*n*) = number of experiments.

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