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Regulation of Neuropeptide Y Release by Neuropeptide Y Receptor Ligands and Calcium Channel Antagonists in Hypothalamic Slices

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Abstract: Neuropeptide Y (NPY) is an important regulator of energy balance in mammals through its orexigenic, antithermogenic, and insulin secretagogue actions. We investigated the regulation of endogenous NPY release from rat hypothalamic slices by NPY receptor ligands and calcium channel antagonists. High-potassium stimulation (60 m*M*) of the slices produced a calcium-dependent threefold increase in NPY release above basal release. The Y2 receptor agonists NPY(13–36) and *N*-acetyl[Leu^{28},Leu^{31}]NPY(24–36), the Y4 agonist rat pancreatic polypeptide (rPP), and the Y4/Y5 agonist human pancreatic polypeptide (hPP) significantly reduced both basal and stimulated NPY release. NPY(13–36)-induced reduction of NPY release could be partially prevented in the presence of the weak Y2 antagonist T4-[NPY(33–36)]$_4$, whereas the hPP- and rPP-induced inhibition of release was not affected by the Y5 antagonist CGP71683A or the Y1 antagonist BIBP3226. The selective Y1, Y2, and Y5 antagonists had no effect on either basal or potassium-stimulated release when administered alone. The calcium channel inhibitors ω-conotoxin GVIA (N-type), ω-agatoxin TK (P/Q-type), and ω-conotoxin MVIIC (Q-type) all significantly inhibited potassium-stimulated NPY release, without any effect on basal release, whereas nifedipine had no effect on either basal or stimulated release. Addition of both ω-conotoxin GVIA and ω-agatoxin TK together completely inhibited the potassium-stimulated release. In conclusion, we have demonstrated that NPY release from hypothalamic slices is calcium-dependent, involving N-, P-, and Q-type calcium channels. NPY release is also inhibited by Y2 agonists and rPP/hPP, suggesting that Y2 and Y4 receptors may act as autoreceptors on NPY-containing nerve terminals.
Key Words: Neuropeptide Y—Calcium channels—Autoreceptors—Y2 receptors—Y4 receptors.
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Neuropeptide Y (NPY), which is found in high concentrations in the hypothalamus of both rats (Allen et al., 1983) and humans (Adrian et al., 1983), has been proposed to play a key role in the regulation of energy balance through its ability to stimulate feeding (Stanley et al., 1986, 1996), inhibit brown adipose tissue thermogenesis (Billington et al., 1991; Egawa et al., 1991), and stimulate insulin secretion (Zarjevski et al., 1993). The effect of both single and multiple NPY injections into the hypothalamus of rodents is to cause a shift towards positive energy balance through increased caloric intake and the shift to energy storage, as fat (Zarjevski et al., 1993; Vettor et al., 1994).

In situations of negative energy balance, such as during periods of starvation or food restriction, when net energy demands are not met with equal energy intake, as food, there is an increase in neuronal activity in NPY-containing neurones in the hypothalamic arcuate (ARC) nucleus (Sahu et al., 1988; Brady et al., 1990). ARC NPYergic neurones, which largely project to the paraventricular and dorsomedial nuclei (Bai et al., 1985; Broberger et al., 1998), release NPY at their terminal boutons, which interact at numerous NPY receptor subtypes, notably Y1 and Y5 receptors, to increase food intake (Currie and Coscina, 1995; Gerald et al., 1996; Schaffhauser et al., 1997; Wieland et al., 1998). For these reasons, the hypothalamic NPY system has been proposed as a protection system against potentially life-threatening loss in energy stores, by stimulating appetite and reducing energy disposal as thermogenesis (King and Williams, 1998).

A dysregulation of the hypothalamic NPY system has been proposed in several pathological and pathophysiological states (King and Williams, 1998), for example, cancer cachexia, in which there is a failure to match energy loss by growing tumours with increased food intake and obesity, when a reduction in leptin receptor activity may lead to overactivity of the hypothalamic

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Abbreviations used: ARC, arcuate; hPP, human pancreatic polypeptide; NPY, neuropeptide Y; rPP, rat pancreatic polypeptide.

NPY system (Chance et al., 1996), as has been demonstrated in genetically obese *db/db* mice and *fa/fa* Zucker rats (Dryden et al., 1995). To understand the factors regulating hypothalamic NPY release, we have studied rat hypothalamic slices, to examine for the possibility that NPY release may be regulated by autoreceptors. Furthermore, we have studied whether NPY release is calcium-dependent and which calcium channel subtype may play a role in the NPY release.

MATERIALS AND METHODS

Adult male Wistar rats (weighing 250–400 g; Liverpool University Biomedical Services) were killed between 09:00 and 10:30 h by a rising concentration of atmospheric CO_2, and their brains were removed. Hypothalamic blocks, bordered by the optic chiasma, mammillary bodies, and amygdaloid sulcus, were quickly dissected and placed in cold (4°C) Krebs bicarbonate buffer (140 m*M* NaCl, 5 m*M* KCl, 2.5 m*M* $CaCl_2$, 1.2 m*M* $MgSO_4$, 5 m*M* glucose, and 25 m*M* $NaHCO_3$, pH 7.4), which had been previously gassed with a 95% O_2/ 5% CO_2 mixture. Slices from each rat were cross-chopped by hand, using a razor blade, to produce slices of ~0.5 mm^3 and then transferred to 0.4 ml of fresh warm Krebs buffer (37°C) in 4-ml scintillation tubes for a preincubation period of 45 min, during which the Krebs buffer was replaced three times. Hypothalamic slices were then incubated for a total of eight 15-min periods in 0.4 ml (37°C): four periods to measure basal release in normal Krebs buffer, followed by a period in high-potassium Krebs buffer (60 m*M* KCl, KCl substituted isosmotically for NaCl) and three recovery periods in normal Krebs buffer. Drugs and peptides were added to the medium at basal incubation period 3 through to the end of the experiment. At the end of the experiment, slices were placed in 400 μl of normal Krebs buffer and sonicated (30 s) to disrupt the tissue. The tissue NPY content and NPY released from the slices were measured by radioimmunoassay using ^{125}I-NPY (2,200 Ci/mmol; Amersham, Bucks, U.K.) and high-affinity rabbit polyclonal anti-NPY antibodies that recognize the N-terminal portion of NPY: cross-reactivity, porcine NPY = 100%, human/rat NPY = 100%, [Leu^{31},Pro^{34}]NPY = 100%, NPY(13–36)NPY < 0.001%, and human/rat pancreatic polypeptide (hPP and rPP, respectively) < 0.001%. Binding of the anti-NPY antibodies to NPY was not affected by the Y2 antagonist T4-[NPY(33–36)]$_4$ (Grouzmann et al., 1997) or the nonpeptide NPY antagonists CGP71683A, BIBP3226, and BIBO3304 (Entzeroth et al., 1995; Criscione et al., 1998; Wieland et al., 1998). Release of NPY from hypothalamic slices is expressed as picograms per hypothalamus per 15 min. The effect of drugs and peptides on the basal release was calculated as the difference between the mean NPY concentrations in periods 1 and 2 versus those in periods 3 and 4. Stimulated release was measured by subtraction from the NPY concentration in period 5 (in high potassium concentration) of that in the mean basal incubation periods, 3 and 4.

Statistical analysis

Differences between control basal and stimulated release and the effect of NPY ligands and calcium channel antagonists were calculated by ANOVA followed by Bonferroni-modified *t* tests for multiple comparisons.

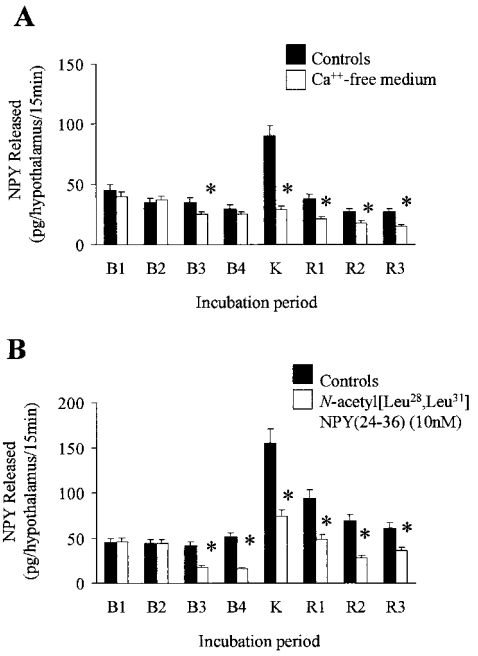

FIG. 1. A: NPY release from hypothalamic slices in Krebs buffer containing calcium (solid columns) or in the absence of external calcium (open columns). Data are mean ± SEM (bars) values for six experiments. **B:** Effect of 10 n*M* *N*-acetyl[Leu^{28},Leu^{31}]NPY(24–36) on basal and potassium-stimulated NPY release from hypothalamic slices. Data are mean ± SEM (bars) values for experiments carried out on six hypothalami. *$p < 0.05$ as compared with controls.

RESULTS

NPY release from the hypothalamic slices could be increased approximately threefold when the tissue was incubated in the high-potassium depolarizing buffer, which then recovered to basal levels over the subsequent recovery period (Fig. 1). Basal and stimulated NPY release was dependent on the presence of extracellular calcium, because removal of calcium from the Krebs buffer resulted in a 30% reduction in basal NPY release and a complete inhibition of the stimulated release (Fig. 1). There was a large amount of NPY remaining in the tissue at the end of the experiment (between 15 and 20 ng per hypothalamus), revealing that only a small percentage of NPY is released during the basal periods (1.9%) and stimulation periods (3.4%).

Addition of the Y2 agonists NPY(13–36) (1 n*M*–1 μ*M*) and *N*-acetyl[Leu^{28},Leu^{31}]NPY(24–36) (1–10 n*M*) resulted in a significant, dose-dependent reduction in both basal and stimulated release (Figs. 1 and 2), as did the Y4/Y5 agonist hPP (10 n*M*–1 μ*M*) and the Y4 agonist rPP (10–100 n*M*; Fig. 3). The NPY(13–36) (100 n*M*)-mediated reduction in basal and stimulated release was partially prevented by addition of the weak Y2 antagonist T4-[NPY(33–36)]$_4$ (1 μ*M*; Fig. 2).

Inhibition of NPY release induced by hPP (50 and 100 n*M*) was not affected by addition of the potent Y5 antagonist CGP71683A (1 μ*M*; Fig. 3) or the potent and

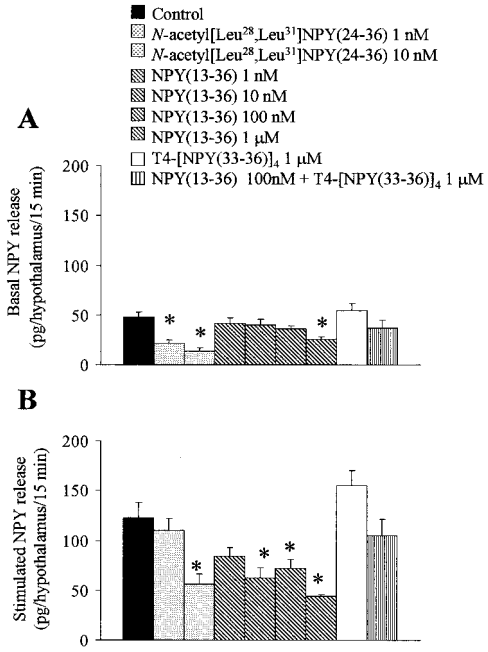

FIG. 2. Effect of *N*-acetyl[Leu^{28},Leu^{31}]NPY(24–36) (1 and 10 n*M*) and NPY(13–36) (1 n*M*–1 μM) on (**A**) basal or (**B**) potassium-stimulated NPY release from hypothalamic slices and the effect of a Y2 antagonist, T4-[NPY(33–36)]$_4$ (1 μM). Data are mean ± SEM (bars) values for between four and six experiments. *$p < 0.05$ as compared with controls.

selective Y1 antagonist BIBO3304 (1 μM; data not shown). The Y2 antagonist T4-[NPY(33–36)]$_4$ (Fig. 2), the Y5 antagonist CGP71683A (Fig. 3), or the Y1 antagonists BIBP3226 and BIBO3304 (all 1 n*M*–1 μM; data not shown) did not affect either basal or stimulated NPY release when added alone.

The calcium channel subtype participating in the NPY release was examined using 100 n*M* nifedipine (L-type), ω-conotoxin GVIA (N-type), ω-agatoxin TK (P/Q-type), and ω-conotoxin MVIIC (Q-type). None of the subtype-selective calcium channel antagonists significantly altered basal NPY release, but ω-conotoxin GVIA, ω-agatoxin TK, and ω-conotoxin MVIIC all significantly inhibited the stimulated NPY release by 44, 61, and 21%, respectively (Fig. 4). Subtraction of the degree of inhibition of potassium-stimulated release by ω-agatoxin TK and ω-conotoxin MVIIC reveals the contribution played by P-type calcium channels (40% of inhibition). Coaddition of ω-conotoxin GVIA and ω-agatoxin together (both 100 n*M*) to block N-, P-, and Q-type calcium channels completely inhibited the potassium-stimulated NPY release (Fig. 4).

DISCUSSION

The large amount of NPY remaining in the tissue at the end of the experiment, coupled with data demonstrating that only a small percentage of NPY is released following exposure of the slices to a high-potassium solution, suggests that this in vitro system may model the physiological NPY release. Although a relatively high potassium depolarizing concentration was used in these studies (60 m*M*), previous work in our laboratory could not reproducibly demonstrate significant stimulated NPY release above the basal release with a lower concentration of KCl (20 m*M*).

We have demonstrated that NPY is released from hypothalamic slices in a calcium-dependent manner that involves N-, P-, and Q-type calcium channels. Depolarization-evoked entry of calcium into nerve terminals plays a key role in triggering neurotransmitter release. These data demonstrate that NPY release does not appear to result from a gradual damage of NPY nerve terminals during the experimental period that may cause a large proportion of the tissue NPY to be released into the incubation medium. The relative contribution of each of the calcium channels on NPY release, as revealed by the channel antagonists, demonstrated that ~40% of the release was due to calcium entry through N-type channels and 40% through P-type channels. The remaining 20% of potassium-stimulated NPY release from hypothalamic slices could be attributed to calcium entry through Q-type channels. Numerous preparations have demonstrated the importance of different calcium channels in the regulation of neurotransmitter release from depolarized nerve terminals. For example, in postganglionic sympathetic nerve terminals, calcium entry is mainly through N-type channels (Maggi et al., 1988; Wright and Angus, 1996) to produce norepinephrine

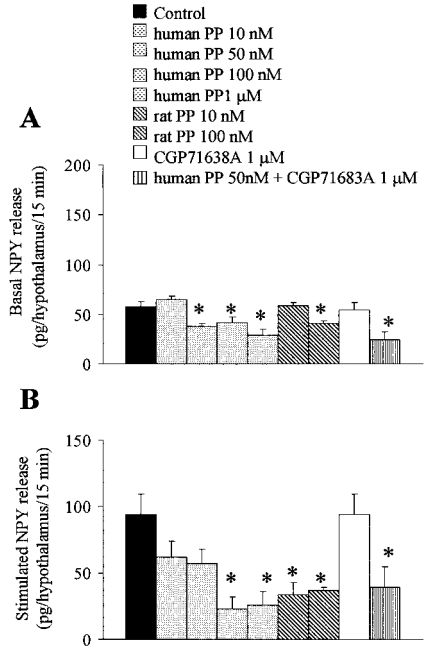

FIG. 3. Effect of hPP (10 n*M*–1 μM) and rPP (10 and 100 n*M*) on (**A**) basal or (**B**) potassium-stimulated NPY release from hypothalamic slices and the effect of the Y5 antagonist CGP71349A (1 μM). Data are mean ± SEM (bars) values for between four and six experiments. *$p < 0.05$ as compared with controls.

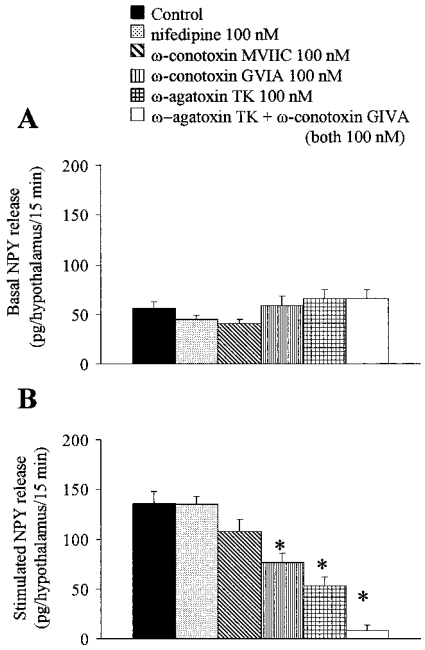

FIG. 4. Effect of the calcium channel antagonists nifedepine, ω-conotoxin MVIIC, ω-conotoxin GVIA, and ω-agatoxin TK (all 100 n*M*) on (**A**) basal or (**B**) potassium-stimulated NPY release from hypothalamic slices. Data are mean ± SEM (bars) values for six experiments. $^*p < 0.05$ as compared with controls.

release. However, there is also a significant proportion of calcium entry through other channels, such as the P and Q subtypes in these postganglionic nerves (Wright and Angus, 1996; Waterman, 1997). The significance of calcium entry into nerve terminals through multiple channel subtypes is presently unknown.

We (Widdowson et al., 1997) and others (Dumont et al., 1990, 1996; Broberger et al., 1997; Gehlert and Gackenheimer, 1997) have demonstrated multiple NPY receptor subtypes in rat hypothalamus located in the ARC, the source of the majority of NPY-containing neurones in the hypothalamus (Bai et al., 1985; Moga and Saper, 1994; King and Williams, 1998) and in many of the ARC NPYergic projection fields (Bai et al., 1985). Within the ARC, binding (Dumont et al., 1990, 1996; Broberger et al., 1997; Widdowson et al., 1997; P.J.K., unpublished data), in situ hybridization (Larsen et al., 1993; Gerald et al., 1996), and immunohistochemical (Larsen et al., 1993) studies have demonstrated the expression and localization of Y1, Y2, Y4, and Y5 receptors. These studies suggest that one or more of these receptor subtypes may act as autoreceptors on NPY-containing neurones. This hypothesis is strengthened by the finding that short NPY-containing projections are localized within the ARC, suggesting that NPY may be released locally to alter neuronal firing (Meister et al., 1989). We have demonstrated that activation of Y2 agonists, such as NPY(13–36) and *N*-acetyl[Leu^{28},Leu^{31}]NPY(24–36) (Potter et al., 1994), can inhibit basal and stimulated NPY release and that this effect can be partially reversed with a Y2 antagonist, T4-[NPY(33–36)]$_4$ (Grouzmann et al., 1997). These data suggest that Y2 receptors may act as presynaptic autoreceptors, to inhibit the further release of NPY from nerve terminals.

We have also demonstrated that both hPP and rPP can also inhibit basal and stimulated NPY release with a pharmacological profile suggesting a role for Y4 presynaptic receptors (Bard et al., 1995; Gehlert et al., 1997; Walker et al., 1997). Our inability to attenuate hPP-induced inhibition of NPY release by the Y5 antagonist CGP71683A suggests that the effects of hPP must be mediated by Y4 and not Y5 receptors. However, the lack of selective antagonists for Y4 receptors does not allow us to conclude the existence of Y4 autoreceptors on NPY-containing nerve terminals. Although the nonselective antagonist 1229U91 displays a high affinity for rat and human Y4 receptors, in addition to Y1 receptors, functional studies indicate that this peptide may act as a full or partial agonist at Y4 receptors (Matthews et al., 1997).

Blockade of presynaptic autoreceptors might have been expected to augment the stimulated NPY release because the localized synaptic concentrations of NPY are prevented from producing a negative feedback on peptide release. None of the antagonists, namely, T4-[NPY(33–36)]$_4$ (Grouzmann et al., 1997), CGP71683A (Criscione et al., 1998), or BIBP3226 or BIBO3304 (Entzeroth et al., 1995; Jacques et al., 1995; Wieland et al., 1998), was able to alter either basal or stimulated NPY release, when administered alone. This suggests that Y1, Y2, and Y5 receptors may not act as autoreceptors, or that the affinity of the weak Y2 antagonist T4-[NPY(33–36)]$_4$ may not be sufficiently high to block fully putative presynaptic Y2 receptors, which is highly probable because the Y2 antagonist displays only a low affinity for Y2 receptors [0.3 μM (Grouzmann et al., 1997)]. Another explanation is that synaptic NPY concentrations may not reach significantly high enough concentrations in our incubation system to activate these autoreceptors owing to dissipation of the neurotransmitter within the relatively large volume of the incubation medium.

As the potent Y1/Y5 agonist [Leu^{31},Pro^{34}]NPY fully cross-reacts with the antibody used in our radioimmunoassay, we have been unable to stimulate selectively Y1 receptors using this ligand, and so we do not have conclusive proof that Y1 receptors do not play a role in regulating NPY release. Y1 receptors are expressed and localized at low levels in the ARC and throughout NPY terminal projection fields in rat hypothalamus (Larsen et al., 1993; Broberger et al., 1997; Gehlert and Gackenheimer, 1997; Widdowson et al., 1997). However, dual-labelling immunohistochemical studies have demonstrated that Y1 receptors are localized in non-NPY containing neurones within the ARC (Broberger et al., 1997). Both findings argue against Y1 receptors as autoreceptors, in support of our data.

In conclusion, we have provided evidence to demonstrate that calcium-dependent, potassium-stimulated NPY release from rat hypothalamic slices involves N-, P-, and Q-type calcium channels. Furthermore, we have provided evidence to suggest that NPY release is also regulated by Y2 and Y4 receptors, which may act as presynaptic autoreceptors.

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