Regulation of Neurotransmission in the Arcuate Nucleus of the Rat by Different Neuropeptide Y Receptors

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We examined the effects of peptides of the neuropeptide Y (NPY)/pancreatic polypeptide (PP) family on synaptic transmission in the arcuate nucleus in rat hypothalamic slices. Application of NPY produced two effects. In some cells NPY produced an outward current that had the properties of a K^+ current. NPY also inhibited the evoked glutamatergic EPSC recorded in these arcuate neurons by a presynaptic mechanism. Although the effects of NPY on the K^+ current reversed within a few minutes of washout of the peptide, its effects on the EPSC frequently were longer lasting (>30 min). Similar effects were observed using peptide YY or the NPY analog [Leu³¹, Pro³⁴]NPY. Although K^+ current activation by [Leu³¹, Pro³⁴]NPY

was blocked by the selective Y1 antagonist BIBP 3226, inhibition of the EPSC was blocked only partially. Other NPY-related peptides such as NPY(13–36), PP, and [D-Trp³²]NPY also inhibited the EPSC. However, none of these peptides produced activation of the K^+ current. Thus, activation of more than one NPY receptor produces synaptic inhibition in the arcuate nucleus. A Y1 receptor activates a K^+ current postsynaptically, and several receptor types appear to inhibit the EPSC by a presynaptic mechanism.

Key words: neuropeptide Y; neuropeptide Y receptors; arcuate nucleus; hypothalamus

Neuropeptide Y (NPY) is a 36 amino acid peptide that is very widely distributed in the CNS and PNS (Colmers and Wahlestedt, 1993). Administration of NPY directly into the CNS produces a host of effects, consistent with its extensive distribution. These effects include cardiovascular, neuroendocrine, and hyperphagic actions (Colmers and Wahlestedt, 1993; Hendry, 1993; Colmers and Bleakman, 1994; Grundemar and Hakanson, 1994; Larhammer, 1996). Furthermore, NPY-deficient mice show a tendency to exhibit seizures (Erickson et al., 1996a), consistent with a possible role for NPY in the control of hippocampal excitability (Colmers et al., 1988; Bleakman et al., 1993; Colmers and Bleakman, 1994).

It is now known that NPY produces its effects through the activation of at least six receptors (Grundemar et al., 1991b; Herzog et al., 1992; Colmers and Bleakman, 1994; Grundemar and Hakanson, 1994; Bard et al., 1995; Gerald et al., 1995, 1996; Hu et al., 1996; Weinberg et al., 1996). Activation of these G-protein-linked receptors leads to various signal transduction events that might potentially produce alterations in neuronal activity. For example, NPY has been shown to inhibit adenylate cyclase and voltage-sensitive Ca^{2+} channels and to activate phospholipase C in a number of cell types (Perney and Miller, 1989; Shigeri and Fujimoto, 1992; Foucart et al., 1993). Furthermore, cloned NPY receptors can produce all of these effects in heterologous expression systems (Herzog et al., 1992; Bard et al., 1995; Gerald et al., 1995, 1996; Sun et al., 1996), and in addition can also produce activation of G-protein gated inwardly rectifying K^+ (GIRK) conductances (Brown et al., 1995; Rimland et al., 1996;

Sun et al., 1996). However, there is only one report of this latter type of response to NPY in neurons—in the frog sympathetic nervous system (Zidichouski et al., 1990). No responses of this type have ever been reported in the CNS.

Injections of small amounts of NPY into the brain stimulate food intake quite prodigiously (Kalra and Kalra, 1996; Miller and Bell, 1996). It appears that these effects are mediated by NPY receptors in the hypothalamus. Precisely which subtype or subtypes of NPY receptors are involved in this response is still unclear, although recent evidence suggests that the Y5 receptor plays a major role in the rat (Gerald et al., 1996; Kalra and Kalra, 1996; Matos et al., 1996; Miller and Bell, 1996).

It is believed that NPY-containing neurons, which project from the arcuate nucleus to the paraventricular nucleus and also send collaterals back into the arcuate, may normally play an important role in the control of feeding behavior and in neuroendocrine regulation (Meister et al., 1989; Erickson et al., 1996a,b; Kalra and Kalra, 1996; Miller and Bell, 1996). These neurons may participate in a negative feedback “lipostat” arrangement whereby they are inhibited by leptin, a cytokine secreted from adipose tissue that inhibits food intake (Stephens et al., 1995; Erickson et al., 1996a,b; Glaum et al., 1996; Miller and Bell, 1996). It is also interesting to note that many of the effects of NPY are of extremely long duration, lasting for extensive periods, even after a single intracerebroventricular injection. These include its well known hyperphagic as well as some of its neuroendocrine and cardiovascular effects (Grundemar et al., 1991a; Huhman and Albers, 1994; Kalra and Kalra, 1996). We have now further investigated the actions of NPY within the arcuate nucleus, with the goal of determining the types of NPY receptors involved and the mechanisms by which its effects are produced.

MATERIALS AND METHODS

Preparation of brain slices. The methods for the preparation of thin brain slices were similar to those described previously (Glaum et al., 1994). Experiments were conducted on Sprague Dawley rats of either sex, aged

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Figure 1. Application of NPY and [Leu³¹,Pro³⁴]NPY activated an outward current in a population of arcuate nucleus neurons. *A*, Application of 100 nM NPY to an arcuate nucleus neuron produced an outward current that readily reversed after washout of the peptide. *B*, Application of 100 nM [Leu³¹,Pro³⁴]NPY also produced a reversible outward current in arcuate nucleus neurons. Traces are scanned images from a chart recording taken from the experiment. Downward deflections represent synaptically evoked currents (0.1 Hz) (*A*) or spontaneous synaptic currents (*B*) and illustrate the relative irreversibility of the effects of [Leu³¹,Pro³⁴]NPY on these currents, compared with its effects on activating the outward current (see text).

10–25 d postnatal. Animals were anesthetized with ether by inhalation and killed by decapitation using a guillotine. The brain was removed rapidly by dissection and placed in chilled (0–6°C) extracellular solution of the following composition (in mM): 126 NaCl, 3.3 KCl, 2.5 CaCl₂, 1.3 MgSO₄, 1.25 NaH₂PO₄, 26 NaHCO₃, and 10 D-glucose (gassed with 95% O₂/5% CO₂, pH 7.4; osmolality = 310 mOsm). Thin (175–200 μ m thick) coronal slices of the arcuate nucleus of the hypothalamus were cut using a vibrating tissue chopper (Vibratome). Slices were maintained at 30–32°C until needed for recording.

For recording, slices were transferred to a submersion chamber mounted on the stage of an upright microscope (Leitz Laborlux) and viewed with a Zeiss 40 $\times$ water immersion objective (Thornwood, NY) with Hoffman Contrast Optics. The slices were perfused continuously throughout the experiment with extracellular solution at room temperature (20–25°C). All recordings were made from visually identified neurons located in the arcuate nucleus. The area of the arcuate nucleus is rich in NPY-positive neuronal cell bodies.

Patch-clamp recording and synaptic stimulation. Patch-clamp recording pipettes were made from thin-walled borosilicate glass capillaries (DC resistance = 3–8 M Ω when filled with internal solution) using a Flaming-Brown horizontal pipette puller (Sutter Instruments, Novato, CA). In all experiments, electrodes were filled with internal solution of the following composition, (in mM): 145 potassium gluconate, 2 MgCl₂, 5 K₂ATP, 1.1 EGTA, 0.1 CaCl₂, and 5 HEPES, pH = 7.2 (osmolality adjusted to 280–290 mOsm). Patch recording pipettes were mounted in the headstage attached to a stage-mounted three-way hydraulic micromanipulator (Narishige, Tokyo, Japan) and were positioned over the somas of neurons under visual control. Conventional methods for obtaining whole-cell recordings from thin slices (Hamill et al., 1981; Edwards et al., 1989) were used. After the attainment of cell access, transmembrane voltage and current were recorded using an Axoclamp 2B (Axon Instruments, Foster City, CA) amplifier (filtered at 10 kHz) in the discontinuous voltage-clamp mode, stored on computer (Gateway 2000) and via chart recorder (Gould, Glen Burnie, MD), and analyzed using Whole-Cell Patch (Strathclyde Electrophysiology Software).

Bipolar tungsten stimulating electrodes were placed lateral to the arcuate nucleus to activate inputs to arcuate nucleus neurons. In all experiments, stimuli of between 50 and 500 μ sec were used to elicit a synaptic response, which were maintained at a frequency of 0.1 Hz to record the time-dependent effects of drug perfusion. For the purposes of data analysis, 2–3 min of evoked EPSCs were averaged (12–18 EPSCs), and the peak of the averaged EPSC was measured. Cells that responded to drug application with a 20% or greater reduction (a change greater than the 99% confidence limits of the control window mean) were considered to have responded positively.

Recording and analysis of miniature EPSCs (mEPSCs). mEPSCs were recorded from arcuate nucleus neurons at a holding potential of –60 to –80 mV in the presence of 1 μ M TTX, 20 μ M 7-chlorokynurenic acid, and

10 μ M bicuculline to pharmacologically isolate AMPA receptor-mediated mEPSCs. [Leu³¹,Pro³⁴]NPY (100 nM) was used as the NPY receptor agonist in all of these experiments. All drugs were allowed to equilibrate for at least 5 min before the onset of recording. Data were sampled continuously at 10 kHz during the recording period, filtered at 1–2 kHz, and acquired to disc using pClamp software (Axon Instruments). Cells were periodically monitored for changes in access resistance, and cells that exhibited any significant (>15%) changes during the recording period were rejected.

mEPSCs were analyzed using pClamp software. All events were examined visually and accepted or rejected based on subjective visual criteria as well as the objective criteria of amplitude, rise time, and decay time. Events that had an amplitude of >3 pA, rise times of between 200 μ sec and 3 msec, and decay times of between 1 and 30 msec were included in the analysis.

Analyzed data from a 3–5 min recording period (100–600 events) were examined and analyzed further using Prism (Graph Pad, San Diego, CA) and Statmost (Datamost). Cumulative probability plots were constructed

Figure 2. High K⁺ containing external medium caused a shift in the reversal potential of the outward current induced by 100 nM [Leu³¹,Pro³⁴]NPY. [Leu³¹,Pro³⁴]NPY-activated currents were examined in this arcuate nucleus neuron at both normal (3.3 mM, open circles) and high (9.5 mM, closed circles) external K⁺ in the presence of 1 μ M TTX. Data points represent the difference of the current required to clamp the cell at potentials from –20 to –100 mV in the absence and presence of [Leu³¹,Pro³⁴]NPY. The shift in the reversal potential of the [Leu³¹,Pro³⁴]NPY-activated current in normal external K⁺ (–87 mV) and high external K⁺ (–52 mV) was consistent with the calculated change for the Nernst K⁺ reversal potential (28.2 mV).

Figure 3. BIBP 3226 inhibited the activation of the outward current $[\text{Leu}^{31}, \text{Pro}^{34}]\text{NPY}$. *A*, *Top trace*, Application of 100 nM $[\text{Leu}^{31}, \text{Pro}^{34}]\text{NPY}$ induced an outward current in an arcuate nucleus neuron. *Middle trace*, Preapplication of the selective Y1 antagonist BIBP 3226 to the same cell as in the top trace blocked the outward current produced by $[\text{Leu}^{31}, \text{Pro}^{34}]\text{NPY}$. *Bottom trace*, After washout of BIBP 3226 (18 min), an outward current was observed again after application of $[\text{Leu}^{31}, \text{Pro}^{34}]\text{NPY}$. *B*, In a different cell, application of BIBP 3226 during an outward current induced by 100 nM $[\text{Leu}^{31}, \text{Pro}^{34}]\text{NPY}$ immediately reversed the actions of the peptide. Note that the effects of $[\text{Leu}^{31}, \text{Pro}^{34}]\text{NPY}$ on the evoked EPSC were not reversed by BIBP 3226. EPSCs were evoked at a frequency of 0.1 Hz in all experiments.

to visually examine the effects of $[\text{Leu}^{31}, \text{Pro}^{34}]\text{NPY}$ on the amplitude and interval distributions of mEPSCs, whereas amplitude and interval distributions were compared statistically using a Kolmogorov–Smirnov (K–S) test or a Mann–Whitney *U* test. Differences in distributions were considered significant if $p < 0.05$. Data are expressed in mean $\pm$ SEM.

Application of drugs. Drugs were dissolved in distilled water and applied by bath perfusion. The following compounds were used: bicuculline methiodide (Sigma, St. Louis, MO), D-AP5 (RBI), 7-chlorokynurenic acid (Tocris Cookson, St. Louis, MO), TTX (Sigma), human NPY (Sigma and Bachem), NPY-free acid (Bachem, King of Prussia, PA), human $[\text{Leu}^{31}, \text{Pro}^{34}]\text{NPY}$ (Sigma), human PYY (Bachem), porcine NPY 13–36 (Sigma), rat PP (Sigma), and BIBP 3226, which was the generous gift of Mary Walker, Synaptic Pharmaceuticals. Drugs were applied for 5–10 min to obtain a steady-state bath concentration.

RESULTS

Whole-cell patch recordings were obtained from 311 neurons in ~133 preparations of 175–200- μm -thick coronal slices of rat arcuate nucleus. Unless otherwise noted, all experiments were performed at a holding potential of -60 or -70 mV in the presence of bicuculline (10 μM), D-AP5 (10 μM), and 1.5 mM external Mg^{2+} to pharmacologically isolate the AMPA receptor-mediated EPSC (Glaum et al., 1996).

Postsynaptic effects of NPY

NPY (100 nM) activated an outward current (22.0 ± 7.7 pA, 6 of 23 cells) in a population of neurons from which recordings were made (Fig. 1*A*). After washout of the peptide, the current relaxed back to the original baseline over a period of 10–15 min. The “Y1” selective NPY agonist $[\text{Leu}^{31}, \text{Pro}^{34}]\text{NPY}$ (100 nM) produced

effects that appeared identical to those observed with NPY (26.7 ± 5.1 pA, 9 of 44 cells), suggesting that the NPY response was produced, at least in part, by activation of Y1 receptors (Fig. 1*B*). The current, which exhibited inward rectification, reversed at -84.0 ± 3.0 mV ($n = 6$) in normal solutions ($K_o = 3.3$ mM), consistent with the idea that it was caused by the activation of a K^+ conductance. The reversal potential shifted to -50.4 ± 4.6 mV ($n = 6$) when the external K^+ was raised to 9.5 mM, consistent with a shift in the Nernst equilibrium potential for K^+ of 28.2 mV and further supporting the contention that $[\text{Leu}^{31}, \text{Pro}^{34}]\text{NPY}$ directly activated a K^+ conductance postsynaptically (Fig. 2). Inclusion of Ba^{2+} in the perfusate blocked the $[\text{Leu}^{31}, \text{Pro}^{34}]\text{NPY}$ -activated current. In three cells, $[\text{Leu}^{31}, \text{Pro}^{34}]\text{NPY}$ activated a current of 17.1 ± 6.8 pA, which was blocked completely by 100 μM Ba^{2+} ($108 \pm 12\%$, $n = 3$) (data not shown). This block by Ba^{2+} further supports the idea that $[\text{Leu}^{31}, \text{Pro}^{34}]\text{NPY}$ activated a K^+ current.

These postsynaptic effects of $[\text{Leu}^{31}, \text{Pro}^{34}]\text{NPY}$ were inhibited by the selective Y1 antagonist BIBP 3226. This was shown in two ways. After observation of a response to $[\text{Leu}^{31}, \text{Pro}^{34}]\text{NPY}$, addition of BIBP 3226 (1 μM) blocked the effect of a second addition of the peptide (Fig. 3*A*; $n = 2$). That this was not because of desensitization in these instances was shown by partial recovery of the response after washout of the peptide (Fig. 3*A*). Second, if BIBP 3226 was added to the slice during a response to $[\text{Leu}^{31}, \text{Pro}^{34}]\text{NPY}$, the current relaxed back to

baseline (Fig. 3*B*; $n = 2$). Addition of 100 nM PYY, which acts as an agonist at multiple subtypes of NPY receptors, including the Y1 subtype, also activated an outward current of similar magnitude (15.7 ± 3 pA; $n = 3/20$). In two of these cells, application of NPY before PYY application resulted in an outward current averaging 25 pA. The Y2 agonist NPY (13–36) (100 nM; $n = 20$), PP (100 nM; $n = 8$), the Y5 agonist [D-Trp³²]NPY (100 nM and 500 nM; $n = 13$ and $n = 6$; Gerald et al., 1996), and NPY-free acid (100 nM; $n = 10$) never produced an outward current.

In summary, it appears that activation of a Y1 receptor on a population of arcuate neurons produces activation of an inwardly rectifying K⁺ current. Although this response has not been observed previously as the result of activation of NPY receptors in the brain, it has frequently been observed with a number of G-protein-linked receptors and is also consistent with the ability of Y1 receptors to activate GIRK-like K⁺ channels in *Xenopus* oocytes (Brown et al., 1995; Sun et al., 1996). Activation of other types of NPY receptors does not appear to produce this response.

NPY receptor-mediated synaptic depression

To examine potential effects of NPY on synaptic transmission, stimulating electrodes were placed ventrolaterally to the arcuate nucleus, and inputs were stimulated at 0.1 Hz while recording from arcuate neurons. Under the conditions used in these experiments (i.e., in the presence of bicuculline and D-AP5), stimulation of inputs resulted in an EPSC that was completely blocked by AMPA antagonists such as CNQX (Glaum et al., 1996). In many cases (15/23), 100 nM NPY produced a strong inhibition of the EPSC (Fig. 4*A*). Similar effects were also observed using [Leu³¹,Pro³⁴]NPY (100 nM; $n = 16/28$) (Fig. 4*B,C*; also see Figs. 1*B* and 3*B*), PYY (100 nM; $n = 16/18$), NPY (13–36) (100 nM; $n = 10/20$), PP (100 nM; $n = 3/8$), and [D-Trp³²]NPY (100 nM; $n = 7/13$; 500 nM; $n = 5/6$) (Fig. 5*A–D*; also see Fig. 6). Also, 100 nM NPY-free acid was ineffective (Fig. 4*D,E*; $n = 10$). In the latter case, we were able to demonstrate in several instances that in recordings where NPY-free acid was ineffective, subsequent addition of NPY produced a depression of the EPSC (Fig. 4*D*; $n = 6$). The lack of effect of NPY-free acid supports the idea that the effects observed with NPY and related peptides are receptor-mediated. The effects of [Leu³¹,Pro³⁴]NPY were potent. The peptide produced a maximal inhibition of $49 \pm 0.2\%$ of the EPSC, with half-maximal effects occurring at 0.74 nM (Fig. 7). Of particular interest was the time course of the inhibition observed. The amplitude of the EPSC began to decline rapidly within a few minutes after addition of the peptide. In a few instances, the NPY receptor-mediated depression reversed after washout (Fig. 4*C*). In many cases, however, the EPSC remained depressed for many

minutes after washout of the drug. As illustrated in Figure 4*A,B*, for example, application of both NPY and [Leu³¹,Pro³⁴]NPY produced a strong depression of the EPSC that did not wash during the course of the experiment, although in the case of NPY (Fig. 4*A*), a slow recovery was evident at 30 min wash. Such long-lasting depression was observed frequently with all NPY agonists (Fig. 5*A–D*). It is unlikely that this was caused by a lack of washout of the agonist, because the activation of the K⁺ conductance reversed much more rapidly (see above). Indeed, when both phenomena were observed in the same cell, the activation of the outward current could be seen to reverse much more rapidly than the inhibition of the EPSC (e.g., Fig. 1*B*). It is also unlikely that the observed long-lasting depression was an artifact introduced by a change in access resistance. Access was checked routinely at several points during each experiment, and cells that showed any changes were rejected. Finally, it is unlikely that such long-lasting depression is because of significant rundown of the synaptic current. In several cases, the addition of an agonist had no effect on synaptic transmission. In such cases, these cells were recorded from routinely for up to 60 min after agonist application, with little observable rundown in the synaptic current (e.g., Fig. 4*E*). Although a small (<10%) degree of rundown was occasionally observed, this could not account for the level of depression that was observed with NPY agonists, nor would it be consistent with the slow washout that was observed in some cells (e.g., Fig. 4*A*). It should also be stressed that long-lasting depression of the EPSC was not observed using NPY-free acid, demonstrating further that these effects were not merely attributable to “run down” of the EPSC amplitude.

The inhibition of the EPSC is likely to be a presynaptic effect, as has been reported elsewhere for the actions of NPY on synaptic transmission (see Discussion). Given the observed postsynaptic effects of NPY reported here, however, we thought it possible that some of the effects of NPY on transmission might be of postsynaptic origin (see also van den Pol et al., 1996). To examine the site of action of NPY on synaptic transmission, we examined the effect of [Leu³¹,Pro³⁴]NPY on mEPSCs recorded in the presence of TTX. For 5 min before recording, 1 μ M TTX was applied to ensure complete block of synaptic transmission. Afterward, 5–7 min of control data were acquired. Then 100 nM [Leu³¹,Pro³⁴]NPY was applied. After another 5-min equilibration period, 5–7 min of data in the presence of [Leu³¹,Pro³⁴]NPY were acquired. In six of six cells, [Leu³¹,Pro³⁴]NPY had no effect on the amplitude distribution of mEPSCs ($p > 0.05$; 6 of 6 cells; K–S test); however, in one of six cells, [Leu³¹,Pro³⁴]NPY caused a significant increase in the interval distribution ($p < 0.005$; 1 of 6 cells; K–S test). Data from the cell exhibiting a significant change in the interval

Figure 4. NPY and [Leu³¹,Pro³⁴]NPY caused a depression of AMPA receptor-mediated synaptic transmission in the arcuate nucleus, whereas NPY free-acid had no effect. *A*, Application of 100 nM NPY caused a depression in the evoked EPSC, as recorded from an arcuate nucleus neuron. After washout of the drug, the EPSC remained depressed for the entire recording period. *B*, Application of 100 nM [Leu³¹,Pro³⁴]NPY also caused a depression in AMPA receptor-mediated synaptic transmission, an effect that again lasted for the entire recording period. *C*, Application of 100 nM [Leu³¹,Pro³⁴]NPY to a different cell than in *B* caused a depression in AMPA receptor-mediated synaptic transmission that readily reversed after washout of the peptide. For *A–C*, *a* represents the time course of the effects of NPY and [Leu³¹,Pro³⁴]NPY, respectively, on synaptic transmission, whereas *b–d* are averaged EPSCs taken from the respective cells showing EPSCs before drug application (*b*), during drug application (*c*), and after washout of the drug (*d*). The time at which the averages were taken are indicated in *a*. *D*, *E*, NPY-free acid had no effect on synaptic transmission. *a*, Time course plot of the effects of NPY-free acid and NPY on synaptic transmission. Application of 100 nM NPY-free acid had no effect on the evoked synaptic current in *D* or *E*. In *D*, application of 100 nM NPY after washout of NPY-free acid produced a significant depression of the EPSC, whereas in *E*, 100 nM NPY had no effect either. *b–d*, Averaged EPSCs taken from the same cell showing before drug addition (*b*), during the application of NPY-free acid (*c*), and during the application of NPY (*d*). The time during which the averages were taken are indicated in *a*. AMPA receptor-mediated synaptic currents were pharmacologically isolated by including 10 μ M bicuculline and 10 μ M D-AP5 in the external bathing medium.

Figure 5. Different NPY receptor agonists induced long-lasting depression of synaptic transmission in the arcuate nucleus. *A–D*, PYY, NPY (13–36), PP, and [D-Trp³²]NPY all caused a depression in AMPA receptor-mediated synaptic transmission in the arcuate nucleus. Each panel (*A–D*) illustrates the time course (*a*) of the drug effect as well as sample averages of EPSCs (*b–d*) taken from the times indicated in the time course plots (*a*). For other details see Figure 4.

distribution are shown in Figure 8*A,B*. When the data from all six cells were pooled and examined, a significant change in the mean interval of the pooled data was observed ($p < 0.01$, Mann–Whitney U test), with no change in the mean amplitude of the pooled data (Fig. 8*C,D*). In addition, even when the one cell that showed a significant change was excluded from the pooled data, a significant change was still observed ($p < 0.01$, $n = 5$; Mann–Whitney U test). This reduction in frequency, coupled with the lack of change in amplitude distribution, is

consistent with a solely presynaptic action of NPY on synaptic transmission.

We attempted to see whether the inhibitory effect of [Leu³¹,Pro³⁴]NPY on the EPSC was also produced by activation of a Y1 receptor. We found that BIBP 3226 was unable to reverse the effects of NPY agonists as it did with the postsynaptic effects of these peptides (Fig. 3*B*). Depending on the signal transduction system involved, however, this might not necessarily be expected. Thus, we also attempted to see whether addition of BIBP 3226

Figure 6. A histogram demonstrating the degree of synaptic depression produced by the various NPY receptor agonists. Bars represent % control of a population of averaged EPSCs measured in the presence of the respective agonist. Except for NPY-free acid, only cells that responded to the respective agonist were considered in the construction of the histogram (see Materials and Methods). Data represented are means $\pm$ SEM.

before the addition of [Leu³¹,Pro³⁴]NPY blocked the inhibitory actions of this peptide (Fig. 9). BIBP 3226 (1 μ M) alone had no effect on amplitude of the EPSC ($-1.2 \pm 2.2\%$; 26 cells). In some cells it was clear that [Leu³¹,Pro³⁴]NPY was ineffective in the presence of the antagonist but subsequently produced effects after its washout ($25.7 \pm 3.7\%$ inhibition; 7/24 cells) (Fig. 9A). On the other hand, it was also clear that in other cells, [Leu³¹,Pro³⁴]NPY was able to decrease the amplitude of the EPSC even in the presence of BIBP 3226 ($29.1 \pm 4.3\%$ inhibition; 8/25 cells), sometimes producing further inhibition after removal of the antagonist (Fig. 9B). Thus, it appears that a component of the synaptic depression may be caused by an action on Y1 receptors, although other receptors also seem to be involved, and their relative contribution may differ at different synapses.

DISCUSSION

When the hyperphagic and neuroendocrine actions of NPY are considered, the arcuate nucleus and its connections appear to be of central importance (Stanley, 1993; Kalra and Kalra, 1996; Miller and Bell, 1996). Not only is the arcuate nucleus the source of much of the NPY containing innervation of the PVN, but these neurons also send collaterals back into the arcuate, thereby contributing significantly to the NPY innervation of the arcuate itself (Meister et al., 1989). Regulation of the activity of these neurons may be an important way of regulating food intake. Indeed changes in food intake in many different circumstances are known to be associated with changes in the levels of NPY and NPY-precursor gene expression within the arcuate and PVN (Stanley, 1993). It is clearly of interest, therefore, to define the cellular actions of NPY in this region of the brain. Furthermore, considering that NPY can exert its effects through the activation of a family of different NPY receptors, it is also important to know which of these receptors is responsible for effects mediated by NPY.

Although a considerable amount of data describe the effects and mechanism of action of NPY in the PNS, corresponding data in the CNS are much more limited (Colmers and Bleakman, 1994; Grundemar and Hakanson, 1994). In the PNS, NPY reduces synaptic transmission at a number of sympathetic neuroeffector junctions—an effect that is consistent with the widely reported ability of NPY to inhibit neuronal Ca²⁺ channels (Toth et al., 1993; Colmers and Bleakman, 1994). Indeed, activation of several

Figure 7. Dose–response curve to [Leu³¹,Pro³⁴]NPY. Application of progressively higher doses of [Leu³¹,Pro³⁴]NPY (0.1–100 nM) resulted in a dose-dependent decrease in the amplitude of evoked synaptic currents, as recorded from arcuate nucleus neurons. The number of observations at each concentration are indicated in brackets below each point. Data are means $\pm$ SEM.

NPY receptors has been shown to inhibit N-type Ca²⁺ channels in heterologous expression systems (Sun et al., 1996). Activation of NPY receptors in the CNS also suppresses synaptic transmission in a number of brain areas, including the arcuate nucleus (Colmers and Wahlestedt, 1993; Colmers and Bleakman, 1994; Glaum et al., 1996; Obrietan and van den Pol, 1996). Expression of different NPY receptors in frog oocytes also leads to the activation of co-expressed inwardly rectifying K⁺ channels of the GIRK family (Brown et al., 1995; Rimland et al., 1996; Sun et al., 1996)—an effect that is commonly observed with different G-protein-linked neurotransmitter receptors. In spite of these observations, direct activation of K⁺ currents by NPY has not been demonstrated previously in the CNS. However, the experiments reported here clearly demonstrate for the first time that activation of a K⁺ current(s) by NPY can be observed in a population of arcuate neurons.

The K⁺ current response bears many of the hallmarks of a Y1 receptor-mediated effect. Thus, it is mimicked by PYY and [Leu³¹,Pro³⁴]NPY and is blocked by BIBP 3226. BIBP 3226 appears to be very selective for Y1 receptors, in so far as its actions on the diverse family of NPY receptors are concerned (Doods et al., 1995; Wieland et al., 1995; Gerald et al., 1996; Sun et al., 1996). Thus, although it has become clear recently that [Leu³¹,Pro³⁴]NPY activates several types of NPY receptors in addition to Y1, including the recently described rat and murine Y5 receptors (Gerald et al., 1996; Weinberg et al., 1996), block by BIBP 3226 has thus far proven to be specific for Y1 receptors (Rudolf et al., 1994; Wieland et al., 1995; Gerald et al., 1996). We also observed that activation of other types of NPY receptors by their relevant “selective” agonists (e.g., PP, NPY 13–36, [D-Trp³²]NPY) did not activate a K⁺ current in these arcuate neurons. Thus, it appears that a Y1 receptor localized on a population of arcuate neurons is responsible for this effect. The fact that other NPY receptors can also activate K⁺ currents in heterologous expression systems suggests, however, that all NPY receptors may also be capable of producing similar responses in other parts of the brain. The reason that such effects have never been observed previously in the CNS may relate to other factors dictating the selectivity of responses activated by G-protein-linked

Figure 8. [Leu³¹,Pro³⁴]NPY causes a reduction in the frequency of mEPSCs with no effect on the amplitude distribution. *A, B*, Cumulative probability plots of the distribution of mEPSC amplitudes (*A*) and intervals (*B*) for a single cell showing a significant change in the interval distribution ($p < 0.005$; K-S test). *C, D*, Effect of [Leu³¹,Pro³⁴]NPY on the mean amplitude (*C*) and mean interval (*D*) for data pooled from six cells. The pooled data showed a significant change in the interval ($p < 0.01$, Mann-Whitney U test), whereas no significant change in the mean amplitude was observed. All data were acquired in the presence of 1 μ M TTX, 20 μ M 7-chlorokynurenic acid, and 10 μ M bicuculline.

receptors (Schreibmayer et al., 1996). It should also be noted that the identity of the K⁺ current(s) activated by NPY in arcuate nucleus neurons remains to be determined precisely.

The synaptic depression produced by NPY receptor activation in the arcuate is more difficult to characterize. We demonstrated previously that NPY could block AMPA receptor-mediated EPSCs and GABA_A receptor-mediated IPSCs in the arcuate, whereas the cytokine leptin was capable of blocking only EPSCs (Glaum et al., 1996). As we now demonstrate, many of these NPY receptor-mediated effects are extremely long lasting. Such observations appear analogous to those of Obrietan and van den Pol (1996) and van den Pol et al. (1996), who demonstrated that NPY produced a long-lasting suppression of GABAergic transmission in the SCN and also showed that a similarly long-lasting effect seemed to occur with GABAergic transmission in the arcuate (Obrietan and van den Pol, 1996). In addition, van den Pol et al. (1996) recently reported an NPY receptor-mediated long-term depression of excitatory synaptic transmission in the SCN. These

observations may provide an electrophysiological correlate to reports of “long-term” effects of NPY in whole-animal studies (Grundemar et al., 1991a; Huhman and Alders 1994). The mechanisms underlying such long-term synaptic depression are unclear; however, the contention that it is caused by a presynaptic inhibition of release is supported by the observation that the frequency of mEPSCs was reduced in the presence of [Leu³¹,Pro³⁴]NPY, whereas the amplitude distribution remained unchanged. In agreement with these observations, van den Pol et al. (1996) also reported that the frequency of mEPSCs was reduced by application of NPY in the SCN, and McQuiston and Colmers (1996) reported similar effects of NPY in the CA3 region of the hippocampus. Previous studies have demonstrated that NPY suppresses transmitter release at many synapses, primarily through inhibition of Ca²⁺ channels such as N-type channels (Toth et al., 1993; Colmers and Bleakman, 1994; Chen and van den Pol, 1996; McQuiston et al., 1996). Thus, inhibition of Ca²⁺ channels could play a role in the observed presynaptic inhibition; however, our

Figure 9. BIBP 3226 was capable of blocking some, but not all, of the effects produced by $[Leu^{31},Pro^{34}]NPY$. *A*, $1 \mu M$ BIBP 3226 completely blocked the actions of $100 nM$ $[Leu^{31},Pro^{34}]NPY$. However, after washout of BIBP 3226, there was a reduction in the amplitude of the evoked EPSC. *B*, BIBP 3226 blocked a portion of the response mediated by $[Leu^{31},Pro^{34}]NPY$. Application of $[Leu^{31},Pro^{34}]NPY$ in the presence of BIBP 3226 resulted in a notable (20%) reduction in the amplitude of the EPSC. After washout of BIBP 3226 from the slice, there was a further reduction in the EPSC amplitude. For both *A* and *B*, *a* represents the time course of the effects of $[Leu^{31},Pro^{34}]NPY$ in the presence and absence of BIBP 3226, whereas *b–e* are averaged EPSCs taken from the respective cell in control conditions (*b*), in the presence of BIBP 3226 (*c*), in the presence of BIBP 3226 and $[Leu^{31},Pro^{34}]NPY$ (*d*), and in the presence of $[Leu^{31},Pro^{34}]NPY$ only, after washout of BIBP 3226 (*e*).

observations that $[Leu^{31},Pro^{34}]NPY$ reduced the frequency of mEPSCs in the presence of TTX also suggests that some additional site of action might be involved. Similar types of effects have

been observed in other instances of presynaptic inhibition (e.g., Scholz and Miller, 1992; van den Pol et al., 1996) and may be indicative of neurotransmitter acting directly on the release apparatus or a similar site.

It appears that more than one type of NPY receptor can mediate suppression of the EPSC (Chen and van den Pol, 1996). The response is produced by NPY and $[Leu^{31},Pro^{34}]NPY$ and can be blocked by BIBP 3226 in some instances, indicating the involvement of a Y1 receptor in at least some of the responses. BIBP 3226, however, could not completely prevent the effects of $[Leu^{31},Pro^{34}]NPY$ in many instances, implying the participation of other NPY receptor subtypes as well. The consistent effects of PYY do not support the view that Y3 receptors are involved. It is interesting to note, however, that $[D-Trp^{32}]NPY$ produced suppression of the EPSC. This compound was described originally as an NPY antagonist (Balasubramanian et al., 1994); however it has been shown recently to be an effective agonist at the rat Y5 receptor (Gerald et al., 1996) and furthermore produces hyperphagic effects (Gerald et al., 1996; Matos et al., 1996). Interestingly, NPY, PYY, and $[Leu^{31},Pro^{34}]NPY$ are all effective agonists at the Y5 receptor, and BIBP 3226 does not act as an antagonist. Additionally, Y5 receptor mRNA was found within the arcuate nucleus (Gerald et al., 1996). These observations therefore suggest a role for the Y5 receptor, in addition to the Y1 receptor, in the suppression of synaptic transmission within the arcuate nucleus. Because of the recent description of so many new subtypes of NPY receptors, it is difficult to make definitive conclusions as to which of these subtypes mediate the inhibitory effects on synaptic transmission. Nevertheless, it is clear that the Y1 and at least one other subtype of NPY receptor are involved. It is interesting to note that an autoreceptor role for Y1 receptors in the arcuate could explain why antisense inhibition of this receptor produces a “paradoxical” increase in feeding behavior (Heilig, 1995).

In conclusion, we have demonstrated the presence of NPY receptor-mediated short- and long-term synaptic modulation within the arcuate nucleus. It appears that NPY inhibits the release of glutamate and GABA within the arcuate nucleus and may additionally regulate its own release. These effects have interesting implications for the reported central actions of NPY. Both types of effects may normally be active in regulating synaptic transmission and may represent mechanisms by which feeding and other behaviors are regulated.

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