Multiple Neuropeptide Y Receptors Regulate K⁺ and Ca²⁺ Channels in Acutely Isolated Neurons From the Rat Arcuate Nucleus

LIHJEN SUN AND RICHARD J. MILLER
Department of Pharmacological and Physiological Sciences, The University of Chicago, Chicago, Illinois 60637

Sun, Lihjen and Richard J. Miller. Multiple neuropeptide Y receptors regulate potassium and calcium channels in acutely isolated neurons from the arcuate nucleus of the rat. J. Neurophysiol. 81: 1391–1403, 1999. We examined the effects of neuropeptide Y (NPY) and related peptides on Ca²⁺ and K⁺ currents in acutely isolated neurons from the arcuate nucleus of the rat. NPY analogues that activated all of the known NPY receptors (Y₁–Y₅), produced voltage-dependent inhibition of Ca²⁺ currents and activation of inwardly rectifying K⁺ currents in arcuate neurons. Both of these effects could occur simultaneously in the same cells. In some cells, activation of Y₄-like currents in arcuate neurons can regulate both Ca²⁺ and K⁺ conductances leading to a reduction in neuronal excitability and a suppression of neurotransmitter release.

INTRODUCTION

Neuropeptide Y (NPY), peptide YY (PYY), and pancreatic polypeptide (PP) constitute a family of homologous neuropeptides that have been suggested as having numerous functions throughout the central and peripheral nervous systems (Colmers and Wahlestedt 1993; Dumont et al. 1992; Tatemoto et al. 1982). One area of considerable current interest is the control of hypothalamic function by these peptides and the regulation of eating behavior in particular (Bek et al. 1993; Jhanwar-Uniyal et al. 1993; Kalra 1997; Miller and Bell 1996; Stanley et al. 1985, 1986; Tomaszuk et al. 1996). Injection of NPY and its analogues into various hypothalamic nuclei strongly stimulates eating, even in previously satiated animals (Stanley and Leibowitz 1985; Stanley et al. 1986). For this and other reasons, it is thought that NPY-containing neurons operating within the hypothalamus normally play a central role in the control of eating behavior.

NPY produces its effects by activating G-protein-linked NPY receptors of which at least five types exist in the rat (Y₁–Y₅) (Bard et al. 1995; Gerald et al. 1995, 1996; Hu et al. 1996; Larhammar et al. 1992; Lundell et al. 1996). Activation of these receptors produces effects on [Ca²⁺], adenylate cyclase, and a number of ion channels (Colmers and Bleakman 1994). Much interest has centered on the identity of the NPY receptor subtype(s) that is responsible for mediating the effects of NPY on eating. Some controversy exists on this point, and data indicating a role for Y₁, Y₅, or some as-yet uncharacterized NPY receptor type have been forthcoming (Gerald et al. 1996; Kanatani et al. 1996; Lopez-Valpuesta et al. 1996; O’Shea et al. 1997; Schaffhauser et al. 1997).

Most of the NPY-containing neurons within the hypothalamus originate in the arcuate nucleus and innervate the paraventricular (PVN) and other nuclei as well as extending collateral connections into the arcuate (Bai et al. 1985; Billington and Levine 1992; Kalra 1997; Meister et al. 1989). This organization suggests that NPY may regulate synaptic communication in different hypothalamic nuclei, including the arcuate nucleus itself. We previously demonstrated with a rat hypothalamic slice preparation that NPY analogues targeting different types of NPY receptors could inhibit both excitatory (glutamate) and inhibitory (GABA)-mediated synaptic transmission in the arcuate nucleus (Glaum et al. 1996; Rhim et al. 1997). In the former case, we demonstrated that activation of all of the major types of NPY receptors could inhibit the evoked release of glutamate. Furthermore activation of postsynaptic Y₁ receptors was shown to activate a K⁺ conductance in arcuate neurons in hypothalamic slices.

To further define mechanisms of NPY action within the arcuate nucleus, we have now examined the effects of NPY on acutely isolated arcuate neurons. We demonstrate that NPY can regulate both Ca²⁺ and K⁺ currents in these neurons and also that this regulation produces a change in the pattern of electrical signaling by these cells.

METHODS

Acute isolation of arcuate neurons

Arcuate neurons were isolated using the techniques modified from Rhim et al. (Rhim and Miller 1994). Rats, aged 10–20 day postnatal, were anesthetized and decapitated. The brain was removed in ice-cold artificial cerebrospinal fluid (ACSF) solution containing (in mM) 126 NaCl, 3 KCl, 2.5 CaCl₂, 1.5 MgSO₄, 1 NaH₂PO₄, 26.2 NaHCO₃, and 10 glucose, pH 7.4; osmolarity ~310 mosmol] gassed with 95% O₂-5% CO₂. The transverse slices (375-mm thick) where the third ventricle extended laterally over the median eminence and infundibular stem were cut coronally using a vibrating tissue chopper (Vibratome) and kept in a holding chamber filled with 31°C ACSF gassed with 95% O₂-5% CO₂. Three to four slices of the hypothalamic arcuate region usually were obtained from one rat.

Slices were treated enzymatically with 15 U/ml papain (preacti-

vated in a Ca²⁺- and Mg²⁺-free ACSF with 3 mM ETDA and 0.16 mg/ml L-cysteine for 30 min) at 31°C for 1 h. After incubation, the
slices were rinsed twice with ACSF containing ovomucoid (trypsin inhibitor). The slices were kept in the holding chamber containing ACSF bubbled with 95% O₂ - 5% CO₂ for 1 h before dissociation. When needed, slices were taken out, and the areas of the arcuate nucleus were micropunched under the dissecting microscope and placed on coverslips. The cells were triturated in plating media (Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and 1% 10,000 µg streptomycin/10,000 U penicillin, pH 7.4, osmolarity ≈ 305) using progressive smaller capillary pipettes. The dissociated neurons were allowed to settle at 37°C for 30 min before recording.

The neurons were heterogeneous in shape and size. The majority of the cells were round, oval, or spindle shaped and measured 12–20 mm in diameter (Fig. 1). Some cells maintained their original morphological features, such as dendritic processes. Most of the recordings were made on cells without long processes.

Electrophysiological recordings of Ca²⁺ currents

The method used for electrophysiological recordings was similar to that previously described (Toth et al. 1996). Recording of Ca²⁺ currents was made using the whole cell voltage-clamp technique. Data were acquired using a Axopatch 1D (Axon Instrument, Foster City, CA) amplifier, filtered at 2 kHz and stored in the computer. Ca²⁺ currents were evoked every 20 s by a 200-ms voltage step from −80 to +10 mV.

Patch electrodes were filled with CsCl-bis-(o-aminophenoxy)-N,N',N’-tetraacetic acid (BAPTA)-based internal solution [which contained (in mM) 100 CsCl, 1 MgCl₂, 10 HEPES, 10 BAPTA, 5 phosphocreatinin, 2 MgATP, and 1 Tris-GTP plus 20 U/ml creatine phosphokinase]. The neurons first were perfused in 2 Na⁺-Ca²⁺ solution [which contained (in mM) 2 CaCl₂, 138 NaCl, 1 MgCl₂, 5 KCl, 10 HEPES, and 10 glucose, pH to 7.4 with NaOH, and osmolarity 305–310], then in a 5Ca²⁺-TEA solution [which contained (in mM) 5 CaCl₂, 144 TEACl, 1 MgCl₂, 10 HEPES, and 10 glucose, pH to 7.4 by TEAOH, and osmolarity 305–310] with and without NPY analogues. NPY (human), PYY (human), [Leu³¹Pro³⁴] NPY (human), hPP (human) (above from Sigma, St. Louis, MO or American Peptide, Sunnyvale, CA), rPP (rat), NPY 13–36 (human, rat), NPY 2–36 (human, rat), C2-NPY (porcine) and [d-Trp³²]-NPY (human, rat) (above from Bachem, King of Prussia, PA) were tested.

Intracellular Ca²⁺ measurement

The method used to measure changes in [Ca²⁺], was similar to that previously described (Rhim and Miller 1994). The acetoxymethyl-ester form of fura-2 (fura-2/AM; Molecular Probes, Table 1. Relative selectivities of NPY and analogues

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Y1</th>
<th>Y2</th>
<th>Y4</th>
<th>Y5</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPY</td>
<td>1</td>
<td>3.6</td>
<td>*</td>
<td>6.9</td>
</tr>
<tr>
<td>NPY 2–36</td>
<td>2.8</td>
<td>1.3</td>
<td>*</td>
<td>1</td>
</tr>
<tr>
<td>NPY 13–36</td>
<td>136.4</td>
<td>1</td>
<td>*</td>
<td>8.3</td>
</tr>
<tr>
<td>[Leu³¹Pro³⁴] NPY</td>
<td>1</td>
<td>*</td>
<td>15.8</td>
<td>11.1</td>
</tr>
<tr>
<td>[d-Trp³²]-NPY</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>1</td>
</tr>
<tr>
<td>C2-NPY</td>
<td>*</td>
<td>1</td>
<td>*</td>
<td>46.8</td>
</tr>
<tr>
<td>PYY</td>
<td>1</td>
<td>1</td>
<td>*</td>
<td>1.7</td>
</tr>
<tr>
<td>hPP (human)</td>
<td>*</td>
<td>*</td>
<td>1</td>
<td>37.8</td>
</tr>
<tr>
<td>rPP (rat)</td>
<td>*</td>
<td>*</td>
<td>1</td>
<td>*</td>
</tr>
</tbody>
</table>

Relative selectivities of neuropeptide Y (NPY) and analogues at different cloned rat NPY receptors compiled from Gerald et al. (1996) and Sun et al. (1998). PYY, peptide YY; hPP, human pancreatic polypeptide; rPP, rat pancreatic polypeptide. *, >1,000.
Eugene, OR) was used as a Ca\textsuperscript{2+} indicator. Acutely dissociated arcuate neurons were allowed to rest for 40 min before the fura-2 experiment. Neurons then were loaded with fura-2 (3 mM) for 25 min, rinsed and allowed 20 min to de-esterify the dye. Intracellular free Ca\textsuperscript{2+}, [Ca\textsuperscript{2+}]\textsubscript{i}, was measured by digital video microfluorimetry using an intensified CCD camera and Universal Imaging software. Cells were illuminated by a 150-W xenon lamp, and excitation wavelengths (340 and 380 nm) were selected by a filter changer. The membrane potential was changed by an application of 50 mM K\textsuperscript{+} solution (with KCl substituted for an equimolar amount of NaCl) via an automatic fast U-tube system. The effects on [Ca\textsuperscript{2+}]\textsubscript{i} produced by different NPY analogues were tested.

**Electrophysiological recordings of K\textsuperscript{+} currents**

The method used for recording of K\textsuperscript{+} currents was modified from Sodickson and Bean (1996). Whole cell voltage-clamp recordings were made using a ramp protocol from -120 or -140 mV to +60 mV of a 100-ms stimulation interval (see Sodickson and Bean 1998). The internal solution was K-based [it contained (in mM) 130 K-gluconate, 15 KCl, 5 MgCl\textsubscript{2}, 10 HEPES, 9 EGTA, 5 phosphocreatinin, 2 MgATP, and 1 Tris-GTP, and 20 U/ml creatine phosphokininase, pH 7.4; osmolality \textasciitilde 285). External solution was either 2 Na\textsuperscript{+}-Ca\textsuperscript{2+} or 30 mM K\textsuperscript{+} solution (with KCl substituted for an equimolar amount of NaCl) with 1 \mu M tetrodo-
TABLE 2. Effects of NPY analogues on Ca\textsuperscript{2+} and K\textsuperscript{+} currents

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Magnitude of Ca\textsuperscript{2+} Current, %</th>
<th>Enhancement of K\textsuperscript{+} Current, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>hPP</td>
<td>64.9 ± 33.0 (4/20)</td>
<td>200.7 ± 34.6 (2/6)</td>
</tr>
<tr>
<td>rPP</td>
<td>72.8 ± 13.2 (2/9)</td>
<td>NA</td>
</tr>
<tr>
<td>PYY</td>
<td>62.3 ± 14.1 (17/28)</td>
<td>175.7 ± 43.4 (10/19)</td>
</tr>
<tr>
<td>NPY</td>
<td>71.6 ± 14.5 (14/22)</td>
<td>192.1 ± 61.5 (11/19)</td>
</tr>
<tr>
<td>[Leu\textsuperscript{30}Pro\textsuperscript{31}]-NPY</td>
<td>72.3 ± 23.2 (25/56)</td>
<td>288.5 ± 152.8 (27/54)</td>
</tr>
<tr>
<td>C2-NPY</td>
<td>61.6 ± 15.6 (3/7)</td>
<td>NA</td>
</tr>
<tr>
<td>NPY 2-36</td>
<td>71.1 ± 18.3 (69)</td>
<td>252.5 ± 104.7 (57)</td>
</tr>
<tr>
<td>NPY 13-36</td>
<td>67.5 ± 18.4 (17/28)</td>
<td>204.0 ± 71.8 (23/53)</td>
</tr>
<tr>
<td>[\textit{\textdagger}Trp\textsuperscript{32}]-NPY</td>
<td>72.4 ± 24.4 (7/17)</td>
<td>271.9 ± 68.3 (4/8)</td>
</tr>
</tbody>
</table>

Effects on Ca\textsuperscript{2+} and K\textsuperscript{+} currents produced by different NPY analogues (see Figs. 2 and 6). The peptide concentration was 100 nM for all analogues except NPY 13–36 (200 nM) and [\textit{\textdagger}Trp\textsuperscript{32}]-NPY (300 nM). Data (means ± SE) indicate the ratio of the maximal peak current amplitude in the presence of agonist to the current amplitude evoked without agonist. Numbers in parentheses indicate the number of cells responding to agonist compared with the total number of cells tested. NA, not available.

Recordings of Ca\textsuperscript{2+} and K\textsuperscript{+} currents in the same neuron

The internal solution used was a K\textsuperscript{+}-based internal solution described in the recording of K\textsuperscript{+} currents. The external solution was alternated between a 5 Ca\textsuperscript{2+}-TEA solution to measure Ca\textsuperscript{2+} currents and 30 mM K\textsuperscript{+} solution with TTX and Cd\textsuperscript{2+} to measure K\textsuperscript{+} currents (Penington and Fox 1994). The recording protocols were identical as described above in the recording of K\textsuperscript{+} currents and recording of K\textsuperscript{+} currents.

Current-clamp recordings

Recordings were made using the whole cell current-clamp method. A K\textsuperscript{+}-based internal solution was used. Neurons were current-clamped at a holding potential of –60 mV. Different current injections (0.1, 0.2, 0.3, 0.4, 0.5, 1, 1.5, and 2 nA) were made. The membrane potential was acquired at 5 kHz and filtered at 2 kHz. Changes in membrane potential produced by NPY analogues also were monitored using a chart recorder (RS-3400, Gould, Cleveland, OH).

Preparation of arcuate nucleus mRNA and first-strand DNA

Areas of the arcuate nucleus were punched out under a microscope and quickly frozen in dry ice, and stored at –80°C. mRNA was extracted (QuickPrep Micro mRNA Purification Kit, Pharmacia Biotech, Piscataway, NJ), followed by first strand cDNA synthesis primed by random hexamers (Superscript Preamplification System, GIBCO, Grand Island, NY).

PCR-Southern hybridization

First-strand cDNA synthesized from arcuate nucleus mRNA as well as receptor cDNAs subcloned into Bluescript (Y1, Y2, and Y4 were generous gifts from Synaptic Pharmaceuticals; Y5 was cloned from a published sequence GenBank U56078) were used as templates in PCR. dNTP, PCR buffer (containing 15 μM MgCl\textsubscript{2}) and AmpliTaq DNA polymerase (5 U/ml) were all obtained from Perkin Elmer (Foster City, CA). The following oligonucleotide primer pairs (forward and reverse) were used:

- **Y1**: 5’ GAAGAACCCTAAGTCCG 3’, and 5’ TCTCAGCAGCTCAAGTCTTGGT 3’;
- **Y2**: 5’ ATGGGTCATTTGAGGGCAGAGA AGA 3’, and 5’ ATGGTGCCTGACTAGAAAAGA 3’;
- **Y4**: 5’ TGAATAC-ATTGGTAGCCTCTGAGAAAGA 3’;
- **Y5**: 5’ TAATGGGACGTCTCTTCTTCT 3’, and 5’ CAGAGGAATCATGACCATGGT 3’.

Arcuate nucleus cDNA, receptor cDNA (as a positive control), and water (as a negative control) were used as templates in separate PCR reactions for each NPY receptor. PCR conditions were 3 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 57°C, and 1 min at 72°C, then 3 min at 72°C for Y1, Y2, and Y4; 3 min at 94°C, followed by 30 cycles of 1 min at 94°C, 30 s at 60°C, and 1 min at 72°C, then 3 min at 72°C for Y5.

Equal volumes of each PCR product were analyzed by agarose gel electrophoresis. PCR products were transferred to a Hybrid-N\textsuperscript{+} membrane (Amersham, Arlington Heights, IL), then hybridized at 42°C overnight with \textsuperscript{32}P-\textalpha-dCTP-labeled receptor cDNA probes, which were made using random priming (Megaprime DNA labeling system, Amersham), followed by purification on Sephadex G-50 columns (NICK DNA grade, Pharmacia Biotech). After hybridization, membranes were washed as previously described (Sambrook et al. 1989; Toth et al. 1996) and then exposed to autoradiography films.
RESULTS

NPY modulation of Ca\(^{2+}\) channels

We examined the effects of NPY and its analogues (Table 1) on the Ca\(^{2+}\) currents in acutely isolated arcuate neurons. In 97 of 202 cells tested, NPY analogues reduced the peak amplitude of the Ca\(^{2+}\) currents by an average of 31.1 ± 2.0% (mean ± SE; Fig. 2). All the peptides examined (see Table 1; concentration 100–300 nM) could inhibit reversibly the Ca\(^{2+}\) currents to a similar extent (Table 2). Variable amounts of kinetic slowing of the Ca\(^{2+}\) currents with each analogue also were observed. In the case of each peptide tested, kinetic slowing was observed in some cases but not others (Figs. 2 and 3). These results suggest that all the known NPY receptors (Y1–Y5) may be present and able to couple to Ca\(^{2+}\) channels in these neurons. The Ca\(^{2+}\) current that was inhibited by NPY analogues was predominantly N type as indicated by its sensitivity to \(\nu\)-conotoxin GVIA (\(\nu\)-CTX). At a concentration of 5–10 \(\mu\)M the toxin inhibited the Ca\(^{2+}\) current in arcuate neurons by 79.3 ± 12.2% \((n = 4)\). When the Ca\(^{2+}\) current was preceded by a depolarizing prepulse to +80 mV, the inhibition produced by NPY and its analogues (PYY, [Leu\(^{31}\)Pro\(^{34}\)]-NPY, and NPY 13–36) was reduced considerably (Fig. 3, 35.9 ± 13.5% inhibition without prepulse, 11.3 ± 7.3% after a prepulse, \(n = 12\), 2–5 cells for each peptide). The relief of inhibition for each of the four peptides was similar, suggesting that they all inhibited the Ca\(^{2+}\) current by a common mechanism.

FIG. 4. Inhibition of Ca\(^{2+}\) currents. A: dose-dependent effects of [Leu\(^{31}\), Pro\(^{34}\)]-NPY and block of these effects by the selective Y1 antagonist BIBP 3226. B: dose-dependent effects of the selective Y2 agonist C2-NPY and inability to block its effects with BIBP 3226. C: dose-dependent effects of the selective Y4 agonist rPP. D and E: cells exhibiting multiple effects of NPY analogues on the same cells.
We attempted to ascertain whether individual arcuate neurons expressed different combinations of NPY receptors by applying a series of NPY analogues sequentially to cells. The analogues used are shown in Table 1. Where possible all peptides were applied in random order to each cell. In some instances, the effects of the selective Y1 antagonist BIBP 3226 were also examined (Doods et al. 1995; Sun et al. 1998; Wieland et al. 1995). As can be seen in Fig. 4, cells responded to different combinations of agonists (n = 60). As also can be seen (Fig. 4, A–C) the effects of the peptides were dose dependent exhibiting half-maximal effects at \( \sim 1.2 \) nM for [Leu\(^{31}, \)Pro\(^{34}\)]-NPY (n = 2), 8.3 nM for C2-NPY (n = 3) and 0.34 nM for rPP (n = 2). Furthermore the effects of [Leu\(^{31}, \)Pro\(^{34}\)]-NPY were blocked selectively by BIBP 3226 (e.g., Fig. 4, A and B). Owing to the selective nature of these compounds at the concentrations employed, the presence of Y1, Y2, and Y4 receptors is clear. Furthermore, the effects of [D-Trp\(^{32}\)]-NPY also suggest the presence of Y5. However, overall, there was no discernible pattern of \( \text{Ca}^{2+} \) current inhibition by different NPY analogues. Different neurons typically responded to one or more NPY analogues.

Intracellular-free \( \text{Ca}^{2+} \) concentration

We further examined the influence of NPY receptors on \( \text{Ca}^{2+} \) signaling in arcuate neurons. All NPY analogues tested were capable of suppressing the \( [\text{Ca}^{2+}]_i \), increase induced by 50 mM K\(^+\) (Fig. 5 and Table 3). These results are consistent with the electrophysiological studies on \( \text{Ca}^{2+} \) currents. Interestingly, however, of 69 cells tested, hPP increased the peak \( [\text{Ca}^{2+}]_i \), in 11 cells and induced oscillations in 18 cells. Oscillations in \( [\text{Ca}^{2+}]_i \) were not produced by any of the other agonists examined except by NPY itself in three instances (Fig. 5).

Modulation of \( \text{K}^+ \) channels

We also studied the regulation of \( \text{K}^+ \) currents by NPY receptors in isolated arcuate neurons. In 82 of 166 cells tested, NPY analogues activated a current with the properties of an inwardly rectifying \( \text{K}^+ \) current (Fig. 6). At a membrane potential of \( -120 \) mV, the amplitude of this current was increased an average of 2.26 ± 0.16-fold. As with the modulation of \( \text{Ca}^{2+} \) currents, all NPY analogues were capable of stimulating \( \text{K}^+ \) currents to similar extents (Table 2). Again, there was no consistent pattern evident in the stimulation of \( \text{K}^+ \) currents when different NPY analogues were applied to single arcuate neurons (Fig. 7).

Modulation of both \( \text{Ca}^{2+} \) and \( \text{K}^+ \) channels in the same neuron

We investigated whether NPY receptors could regulate \( \text{Ca}^{2+} \) and \( \text{K}^+ \) currents in the same isolated arcuate neurons. By changing the external solution from one containing 30 mM K\(^+\) to a 5Ba-TEA solution, \( \text{K}^+ \) current activation and inhibition of \( \text{Ca}^{2+} \) currents could be recorded sequentially in the same cell (Fig. 8). In 6 of 14 cells tested, NPY activated a \( \text{K}^+ \) current and inhibited the \( \text{Ca}^{2+} \) current. In the remainder, we either could not record both \( \text{K}^+ \) and \( \text{Ca}^{2+} \) currents or the cells were not sensitive to NPY analogues.

Changes in firing pattern produced by NPY analogues

How does NPY alter the electrophysiological behavior of arcuate neurons and how are such changes related to the regulation of \( \text{K}^+ \) and \( \text{Ca}^{2+} \) currents? To answer these questions, neurons were held at \(-60 \) mV and induced to fire action potentials by current injection (n = 16). TTX-sensitive Na\(^+\) spikes were induced normally on injecting 0.2- to 0.3-nA currents. With larger current injections (1–2 nA),
Cd$^{2+}$-sensitive Ca$^{2+}$ spikes also were generated. On application of NPY, spiking activity was suppressed at lower magnitude current injections. At higher magnitude current injections, multiple Na$^{+}$ spikes were observed, but Ca$^{2+}$ spikes remained suppressed (Fig. 9).

Membrane potential changes induced by NPY analogues

The membrane potential of a series of cells was held at different values ranging from −20 to −60 mV. In the absence of NPY analogues, cells often showed spontaneous activity including spikes and plateau potentials. After peptide application, the membrane potential hyperpolarized (14 of 25 cells tested) and spontaneous activity ceased (Fig. 10A). The magnitude of the membrane potential change varied from −20 mV to only −5 to −6 mV. In the presence of Cs$^{+}$ (3–5 mM), the change in membrane potential was blocked completely ($n = 2$) or partially ($n = 2$). An example is shown in Fig. 10B. The effect of Cs$^{+}$ suggests that the hyperpolarization induced by NPY analogues might be due to the activation of K$^{+}$ currents.

**TABLE 3.** NPY analogues inhibit Ca$^{2+}$ responses in arcuate neurons

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Inhibition of 50 nM K$^{+}$ [Ca$^{2+}$], Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPY</td>
<td>0.67 ± 0.13 (23/49)</td>
</tr>
<tr>
<td>PYY</td>
<td>0.73 ± 0.6 (5/12)</td>
</tr>
<tr>
<td>NPY 13–36</td>
<td>0.82 ± 0.10 (3/16)</td>
</tr>
<tr>
<td>[Leu$^{31}$Pro$^{34}$]-NPY</td>
<td>0.74 ± 0.5 (15/31)</td>
</tr>
<tr>
<td>[D-Trp$^{32}$]-NPY</td>
<td>0.76 ± 0.7 (6/16)</td>
</tr>
<tr>
<td>hPP</td>
<td>0.80 ± 0.7 (12/69)</td>
</tr>
</tbody>
</table>

NPY analogues reduced the amplitude of 50 mM K$^{+}$-induced [Ca$^{2+}$], increases in arcuate neurons. Data (means ± SE in percent) indicate the ratio of the intracellular [Ca$^{2+}$], response in the presence of agonist to that without agonist (see Fig. 5). Cells not responding to agonists were not included in the calculation. Numbers in parentheses indicate the number of cells responding to agonist compared with the total number of cells tested.

**FIG. 6.** Stimulation of K$^{+}$ currents by NPY receptors in individual arcuate neurons. Recordings were made in 30 mM K$^{+}$ solution and currents were evoked by ramp depolarizations as indicated. A: time course of K$^{+}$ currents activated by NPY 13–36 (200 nM). B: currents at points a and b are shown. C: current/voltage curve obtained by the subtraction of a from b indicating the K$^{+}$ current induced by NPY 13–36. D: reversal potential of the NPY-activated currents as a function of external K$^{+}$. Liquid junction potential was around −5 to −7 mV.
Presence of NPY receptors in the arcuate nucleus

To determine which NPY receptor mRNAs are expressed in the rat arcuate nucleus, we carried out RT-PCR amplification using primers specific for Y1, Y2, Y4, and Y5, followed by Southern blot hybridization using specific probes for each NPY receptor subtype. The expression of all four NPY receptor subtypes (Y1, Y2, Y4, and Y5) could be clearly demonstrated (Fig. 11). Although it is not possible to say from these data whether multiple NPY receptors occur in each cell, it does confirm their presence in the arcuate nucleus as a whole.

**DISCUSSION**

The NPY family of neuropeptides has been shown to produce numerous effects in the hypothalamus that are of great current interest. For example, NPY has been shown to regulate the release of a large number of hormones and to be involved in the control of diurnal rhythms, eating, and other behaviors (Dumont et al. 1992; Grundemar and Hakanson 1994; Huhman et al. 1996; Leibowitz 1991; Tomaszuk et al. 1996). In most instances, however, the precise cellular and anatomic basis for these effects are understood incompletely. The hypothalamus contains large amounts of NPY (Allen et al. 1983; Chronwall et al. 1985; de Quidt and Emson 1986). Several hypothalamic nuclei including the arcuate, suprachiasmatic, periventricular and paraventricular nuclei are densely innervated by NPY immunoreactive fibers, whereas only the arcuate contains a high concentration of NPY immunoreactive perikarya (Allen et al. 1983; Chronwall et al. 1985). It has been established that arcuate NPY neurons innervate several other hypothalamic nuclei and also send collateral fibers into the arcuate itself (Bai et al. 1985; Billington and Levine 1992; Kalra 1997; Meister et al. 1989). Indeed, most of the NPY innervation of the hypothalamus, including that of the arcuate, derives from the arcuate NPY neurons themselves. There are exceptions to this rule and some innervation of the hypothalamus also appears to originate with NPY-containing neurons in the brain stem for example (Sahu et al. 1988; Sawchenko et al. 1985).

Knowledge as to the precise distribution of NPY receptors in the hypothalamus is even scantier. Although some evidence suggests that all of the cloned NPY receptors (i.e., Y1, Y2, Y4, and Y5) are found in this part of the brain (Broberger et al. 1997; Fuxe et al. 1977; Gustafson et al. 1997; Mikkelsen and Larsen 1992; Naveilhan et al. 1997; Widdowson 1997), not a lot is known about either their subcellular localization or individual functions. Some information is available on the distribution of Y1 and Y2 receptors in the arcuate nucleus (Broberger et al. 1997). It appears that, in general, Y1 receptors are...
localized to the pro-opiomelanocortin (POMC)-containing perikarya in the ventrolateral arcuate. In contrast, Y2 receptors appear to be primarily associated with the NPY–containing cell bodies in the ventromedial portion of the nucleus. NPY–containing nerve terminals also are associated with arcuate POMC–containing cell bodies as well as others (Broberger et al. 1997; Garcia de Yebenes et al. 1995). One model therefore has proposed that NPY might act on postsynaptic Y1 receptors to regulate POMC neurons. In addition, the Y2 receptors found in NPY–containing cells might represent presynaptic receptors that regulate NPY release from the terminals of these neurons among other things (Broberger et al. 1997).

Electrophysiological data resulting from the present and previous studies indicate that although this model may be partially correct, it is certainly incomplete. We previously have demonstrated, using a rat hypothalamic slice preparation, that NPY receptors of all types exist presynaptically on both excitatory and inhibitory inputs into the arcuate and that their activation can inhibit glutamate and GABA–mediated synaptic transmission (Glaum et al. 1996; Rhim et al. 1997). We also have shown that NPY can stimulate Y1 receptors postsynaptically, resulting in the activation of an inwardly rectifying K+ current in a subgroup of arcuate neurons. In our previous investigation, we did not observe postsynaptic effects resulting from the activation of other NPY receptor types. In contrast, we have now shown that all four types of cloned NPY receptors exist postsynaptically on arcuate neurons and can couple to K+ and Ca2+ currents. It is conceivable that Y3 receptors (which still have to be cloned) also are present, but there is really no good way of telling. Activation of all of these receptors results in the voltage–dependent inhibition of Ca2+ channels, which in this case are mostly of the N type, and in the activation of K+ channels, which are presumably of the G–protein–activated inwardly rectifying K+ channel (GIRK) family.

One question that arises from the present results is why we can observe activation of K+ currents by all types of NPY receptors using isolated arcuate neurons, when only Y1 agonists produced these effects in the slice (Rhim et al. 1997). One possibility is that some of these receptors normally are located on cell processes away from the cell soma and that these structures are reabsorbed into the cell body on isolation. Another possibility is that our slice recordings were restricted to a particular part of the arcuate (e.g., POMC–containing cells) where the Y1 receptor may be localized selectively and that the isolated cells used in the current studies are the result of a wider sampling of the nucleus. As discussed in the preceding section, previous studies have suggested that different types of NPY receptors may be segregated on different subpopulations of arcuate neurons, although we did not observe any obvious segregation of receptors in the present studies. However, if the isolated cell preparation we used does indeed represent the majority of neuronal types in the arcuate, which is a very complex structure from the neurochemical point of view, then any segregation might not be obvious unless extremely large numbers of cells were examined. Nevertheless, the data clearly show that all types of NPY receptors can exist postsynaptically in the arcuate nucleus—a conclusion that is also consistent with the molecular biological data.

The inhibition of Ca2+ channels we have demonstrated now is a second type of response that would not have been evident from our previous slice studies. As with the K+ channel response, all types of NPY receptors appeared capable of producing this effect and these responses appeared to be "randomly" distributed among cells, consistent with the data on K+ currents discussed before. Indeed, as we now also have demonstrated, both of these types of responses probably occur simultaneously in the same cells. In some respects, such a result is not surprising given that the activation of GIRKs and the voltage–dependent inhibition of N–type Ca2+ channels (presumably the result of the neuronal expression of α1B Ca2+ channel subunits) are both mediated by the βγ subunits of heterotrimeric G proteins (Herlitze et al. 1996; Hille 1994; Huang et al. 1995; Ikeda 1996; Krapivinsky et al. 1995). These G–protein subunits presumably would be released on activation of any type of NPY receptor. The fact that we observe both of these responses (i.e., Ca2+ current inhibition and K+ current activation) together suggests that there is no higher level of hierarchical control of these processes that might result in the

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**FIG. 8.** Activation of K+ currents and inhibition of Ca2+ currents by NPY receptors in the same arcuate neuron. A: activation of K+ currents by [Leu31-Pro34]–NPY. B: subtraction of the 2 curves in A, showing the net increase in K+ current produced by [Leu31-Pro34]–NPY. After removal of the peptide, the external solution was changed to 5Ba–TEA to record Ca2+ currents and the peptide was reapplied. C: resulting inhibition of the Ca2+ current.
observation of one response or the other (Schreibmayer et al. 1996). This potentially could result from the selective localization of some of the molecular elements involved in receptor/ion channel coupling. One caveat, however is that we do not know whether some such regulatory mechanism is disrupted and lost on cell isolation. Ca$^{2+}$ imaging studies also revealed a further response that seemed to be produced by activation of Y4 receptors. In these instances, hPP produced oscillations in [Ca$^{2+}$], suggesting that additional signaling pathways also may be activated by these receptors.
What is the physiological role of the inhibition of Ca\textsuperscript{2+} currents and activation of K\textsuperscript{+} currents? It is likely that some of the cells we recorded from are NPY-containing neurons that project to different parts of the hypothalamus, including the arcuate. The terminals of these neurons probably possess NPY receptors that are important in feedback presynaptic inhibition of transmitter release. Thus the events we have observed in the cell soma may be a manifestation of events that occur in the terminals of these neurons as well. Clearly, the inhibition of Ca\textsuperscript{2+} influx and/or activation of K\textsuperscript{+} conductances may be important mechanisms underlying presynaptic inhibition induced by activation of G-protein-linked receptors (Miller 1998). Inhibition of Ca\textsuperscript{2+} currents by NPY receptors will directly suppress Ca\textsuperscript{2+} influx into terminals. Second, activation of K\textsuperscript{+} channels will increase the conductance of the terminal, making incoming action potentials less effective in activating Ca\textsuperscript{2+} channels. Similar events also may be involved in the presynaptic inhibition of glutamate and GABA release produced by NPY from the terminals of excitatory and inhibitory inputs into the arcuate nucleus (Glaum et al. 1996; Rhim et al. 1997). As we now also have demonstrated, NPY activation of K\textsuperscript{+} currents in the cell bodies of arcuate neurons has a profound inhibitory effect, completely suppressing spiking activity. Thus activation of NPY receptors suppresses arcuate neurons in two ways. First, the excitability of neurons is reduced and then, if spikes do fire, transmitter release from the terminals of the neurons also would be reduced.

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Address for reprint requests: R. J. Miller, Dept. of Pharmacological and Physiological Sciences, The University of Chicago, 947 E. 58th St. (MC 0926), Chicago, IL 60637.

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