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Neuropeptide Y (18–36) Modulates Chromaffin Cell Catecholamine Secretion by Blocking the Nicotinic Receptor Ion Channel[1]

JIALIN ZHENG, RICHARD A. MORRISETT, JIANHUA ZHU[2] and TERRY D. HEXUM

Department of Pharmacology, University of Nebraska Medical Center, Omaha, Nebraska

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ABSTRACT

Neuropeptide Y (NPY) is a widely distributed peptide with varied activities including inhibition of [^{3}H]NE secretion from chromaffin cells. In the present study, we investigated the mechanism through which NPY and NPY fragments inhibit nicotinic receptor induced influx of $^{22}Na^+$ and $^{45}Ca^{++}$ into bovine chromaffin cells. Fragments of NPY, including NPY13–36, NPY18–36 and NPY26–36, are more potent inhibitors of $^{45}Ca^{++}$ and $^{22}Na^+$ influx than NPY. High [K^+]- and BAY K 8644-induced $^{45}Ca^{++}$ influx and veratridine-induced $^{22}Na^+$ influx are not inhibited by either NPY or NPY fragments. Thus, the site of NPY or NPY fragment action is not voltage-gated Ca^{++} or Na^+ channels. A significant amount of acetylcholine-induced $^{45}Ca^{++}$ influx still occurs in the presence of the voltage-gated Ca^{++} channel blockers: nifedipine (L-type), ω-conotoxin-GVIA (N-type) and ω-agatoxin-IVA (P-type). NPY18–36, in the presence of these channel blockers, inhibited the residual nicotinic receptor-induced Ca^{++} influx. The response to NPY18–36 is not pertussis toxin sensitive. The rank orders of potency for inhibition of $^{45}Ca^{++}$ and $^{22}Na^+$ are the same: NPY18–36 ≥ NPY26–36 > NPY13–36 > NPY ≥ $NPY_{free\ acid}$. Moreover, the IC_{50} values for NPY18–36 inhibition of $^{45}Ca^{++}$ influx and $^{22}Na^+$ influx are similar, 0.9×10^{-6} M and 2.03×10^{-6} M, respectively. Regression analysis for inhibition of these two phenomena produced a correlation coefficient of .9697 ($P < .0003$). Regression analysis for inhibition of 1,1-dimethyl-4-phenylpiperizinium (DMPP)-stimulated [^{3}H]NE secretion *vs.* inhibition of DMPP-stimulated $^{22}Na^+$ influx produced a correlation coefficient of .9894 ($P < .0001$). We conclude that NPY modifies nicotinic receptor function by blocking the nicotinic receptor ligand-gated ion channel.

NPY, a member of the pancreatic polypeptide family, can influence various physiologic events, including synaptic transmission. For example, the depolarization-induced release of substance P from rat dorsal root ganglion cells (Walker *et al.*, 1988), acetylcholine from rat nodose neurones (Wiley *et al.*, 1990) and glutamate from rat hippocampus (Colmers *et al.*, 1988) are inhibited by NPY. Moreover, addition of NPY to chromaffin cells in culture results in the inhibition of cholinergic-stimulated [^{3}H]NE secretion (Hexum *et al.*, 1994). In each of these cases, the common finding is that Ca^{++} influx decreases after the addition of NPY. The decrease in Ca^{++} influx resulting in inhibition of transmitter release in the rat tissues is mediated by the G protein-linked NPY receptor (Bleakman *et al.*, 1992; Ewald *et al.*, 1989; Wiley *et al.*, 1990), classified as Y_2 (Bleakman *et al.*, 1991; Colmers *et al.*, 1991). The Y_2 receptor may be coupled to a voltage-dependent calcium channel, *e.g.*, N-type, by a pertussis toxin-sensitive protein such as G_i or G_o.

The mechanism of NPY inhibition of chromaffin cell secretion is characterized by some significant differences. We recently reported that NPY and NPY fragments inhibit nicotinic receptor-stimulated chromaffin cell [^{3}H]NE secretion (Hexum *et al.*, 1994). The inhibition of secretion strongly correlated with the ability of NPY and NPY fragments to inhibit $^{45}Ca^{++}$ influx. However, NPY did not inhibit secretion resulting from the addition of high potassium concentrations, suggesting that calcium channels were not directly influenced by NPY. Moreover, the effect of NPY was not pertussis toxin sensitive. The rank order of potency for the inhibition of secretion and $^{45}Ca^{++}$ influx by NPY and NPY fragments was closely correlated but distinct from that previously reported for effects at Y_1, Y_2 or Y_3 receptors. Based on these findings, we designated the NPY response site as that of a Y_4 subtype. We provide further characterization of NPY inhibition of secretion by using the most potent NPY fragment, NPY18–36. Evidence is furnished that NPY acts by

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[2] Present address: Harvard University, Joslin Diabetes Center, One Joslin Place, Boston, MA 02215.

ABBREVIATIONS: DMPP, 1,1-dimethyl-4-phenylpiperizinium; ACh, acetylcholine; KRH, Krebs-Ringer-HEPES buffer; NE, norepinephrine; NPY, neuropeptide Y; PYY, peptide YY.

blocking ion movement through the nicotinic cholinergic receptor.

Methods

Materials

Collagenase and DNAse were purchased from Sigma Chemical Co. (St. Louis, MO). NPY was obtained from Bachem (Torrance, CA); NPY fragments and related peptides were from Peninsula Labs. Inc. (Belmont, CA). Substituted analogs of NPY(18–36) were provided by Dr. Jean Rivier of The Salk Institute (La Jolla, CA). l-[7,8-^{3}H]noradrenalin ([^{3}H]NE) was purchased from Amersham (Arlington Heights, IL), and $^{45}Ca^{++}$ was purchased from ICN (Irvine, CA). Budget Solve liquid scintillation fluid was from Research Products International Corp (Mount Prospect, IL). Dulbecco's Modified Eagle Medium, fetal bovine serum, Hanks' balanced salt solution and cell culture plasticware were from GIBCO (Gaithersburg, MD). Fluorocarbon filters (pore size 70 μm) were from Spectrum Medical Industries (Los Angeles, CA).

Methods

Cell culture. Isolation and culturing of bovine adrenal chromaffin cells were performed with modifications as previously described (Zhu *et al.*, 1992). Cells were plated in 24-well plastic culture plates at a density of 1×10^6 cells/well in an atmosphere of 5% CO_2 at 37°C.

Secretion. Secretion was studied by measuring the release of [^{3}H]NE from chromaffin cells kept in culture for 3 or more days. Culture media were removed from chromaffin cells by two washings with KRH containing 25 mM HEPES (pH 7.4), 125 mM NaCl, 1.2 mM $CaCl_2$, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4 and 5.6 mM glucose. [^{3}H]NE was added to each well and incubated for 90 min at 37°C in an atmosphere of 5% CO_2. Cells were washed 3 times by incubation at 37°C for 10 min in KRH. Secretion resulted after the addition of stimulating agents dissolved in KRH and incubation for 5 min at 37°C. The effects of peptides were examined by preincubating the cells with the selected peptide for 10 min at 37°C before cell stimulation. At the end of the stimulation period, the incubation media were removed, and the cells were solubilized with 0.25 ml 1 N NaOH. Radioactivity was determined by liquid scintillation spectrometry. Secretion of [^{3}H]NE is expressed as the percentage of cpm in the incubation medium relative to the total cpm (medium plus cellular).

Secretion of endogenous catecholamines was measured by reverse-phase high-performance liquid chromatography with the use of electrochemical detection (Barron and Hexum, 1986). Catecholamines were measured directly in acidified culture medium containing 0.2 mM $NaHSO_3$ and not extracted on alumina.

$^{45}Ca^{++}$ influx. Chromaffin cells were washed twice with calcium free KRH (KRH-Ca^{++}) containing 25 mM HEPES (pH 7.4), 125 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4 and 5.6 mM glucose and incubated (37°C) in KRH-Ca^{++} for 60 min. Calcium uptake was initiated by adding 0.5 ml KRH-Ca^{++} containing 5 μCi $^{45}Ca^{++}$ and either ACh or ACh plus peptide (DMPP was used in some experiments). The reaction was carried out for 10 min at 37°C. The reaction was terminated by the addition of 0.5 ml cold KRH-Ca^{++} containing 10 mM EGTA followed by three washes with the same buffer. Cells were solubilized with 0.4 ml NaOH (1 N) followed by neutralization with HCl (4 N). The radioactivity was measured by scintillation spectroscopy.

$^{22}Na^+$ influx. Chromaffin cells were washed twice with flux buffer (KRH) containing 25 mM HEPES, 1.3 mM $CaCl_2$, 125 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4 and 5.6 mM glucose (pH 7.4) and incubated (37°C) in this buffer for 60 min. The flux buffer was aspirated and replaced with fresh flux buffer containing 2 μCi/ml of $^{22}Na^+$ and 2 mM ouabain with either DMPP or DMPP plus NPY or its fragment, and the reaction was carried out at 37°C in an incubator for 10 min. (The $^{22}Na^+$ flux into cells when the medium did not contain DMPP was assumed to reflect nonreceptor-mediated uptake and was subtracted routinely from values determined in the presence of DMPP.) The reaction was terminated by addition of 0.5 ml cold flux buffer and washed with the same buffer 3 times. Cells were solubilized in 0.4 ml of 1 N NaOH and neutralized with 4 N HCl. The radioactivity was measured by scintillation spectroscopy.

Results

Inhibition of DMPP-induced $^{45}Ca^{++}$ influx by NPY, PYY and NPY fragments. The inhibition of nicotinic receptor-stimulated [^{3}H]NE secretion by NPY correlates closely with the peptide's ability to decrease Ca^{++} influx into chromaffin cells (Hexum *et al.*, 1994). Of the various peptides tested, NPY18–36 was the most effective inhibitor of secretion (fig. 1). The mechanism by which NPY and NPY fragments inhibit nicotinic receptor-stimulated Ca^{++} influx has not been determined. A comparison of increasing concentrations of various peptides, including NPY, NPY18–36, [Leu^{31},Pro^{34}]NPY, $NPY_{free\ acid}$ and PYY, demonstrates that NPY18–36 is the most potent inhibitor of $^{45}Ca^{++}$ influx into chromaffin cells (fig. 1). The IC_{50} for NPY18–36 is 0.9×10^{-6} M.

Effect of Ca^{++} channel antagonists and NPY or NPY18–36 on ACh-induced $^{45}Ca^{++}$ influx. We next examined the extent to which the effect of NPY or NPY18–36 on $^{45}Ca^{++}$ influx was exerted through voltage-gated Ca^{++} channels. NPY inhibited ACh-induced $^{45}Ca^{++}$ influx by 31% after correction for the basal increase in $^{45}Ca^{++}$ influx (fig. 2A). Nifedipine alone, with ω-agatoxin IVA or with ω-agatoxin IVA and ω-conotoxin GVIA (inhibitors of L-, P- and N-type voltage-gated calcium channels, respectively) inhibited ACh-induced $^{45}Ca^{++}$ influx by approximately 59%, 53% and 57%, respectively. The simultaneous addition of all three calcium channel blockers plus NPY resulted in the inhibition of ACh-induced $^{45}Ca^{++}$ influx by 65%. The experiment was repeated using the more potent fragment, NPY18–36, which alone inhibited ACh-induced $^{45}Ca^{++}$ influx by approximately 70% (fig. 2B). Nifedipine alone, with ω-agatoxin IVA or with ω-agatoxin IVA and ω-conotoxin GVIA inhibited ACh-in-

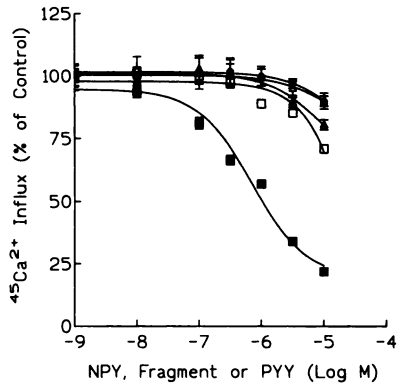

Fig. 1. Inhibition of DMPP-induced $^{45}Ca^{++}$ influx by NPY or NPY fragments. Chromaffin cells were stimulated with DMPP (30 μM) or DMPP and peptide for 10 min at 37°C. NPY or fragments did not alter basal Ca^{++} influx (4320 ± 192 cpm). DMPP stimulation in the absence of peptides is defined as 100% (20,666 ± 452 cpm). Each data point represents the mean ± S.D. of three separate experiments performed in triplicate (basal influx subtracted). NPY (□), NPY18–36 (■), [Leu^{31},Pro^{34}]NPY (△), $NPY_{free\ acid}$ (▲) and PYY (◇).

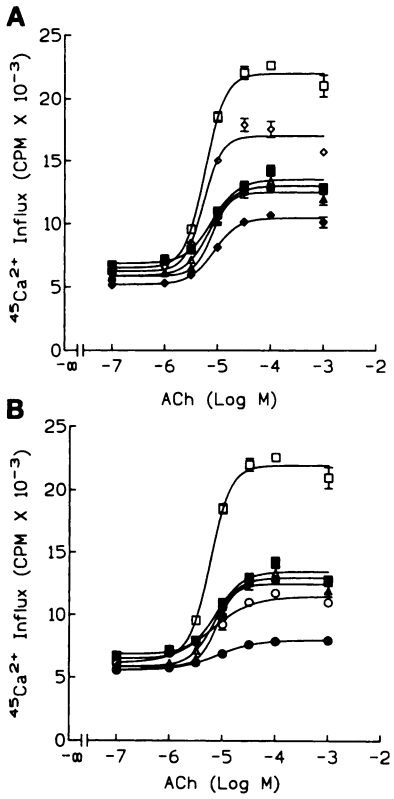

Fig. 2. Effect of NPY (A) or NPY18–36 (B) on ACh-induced influx of $^{45}Ca^{++}$. Increasing ACh concentrations [with atropine (0.5 μM) and physostigmine (10 μM)] were incubated (10 min at 37°C) with chromaffin cells in the presence of the voltage-gated calcium channel blockers nifedipine (50 μM), ω-Agatoxin IVA (0.4 μM) and ω-conotoxin GVIA (2 μM) in the absence or presence of NPY or NPY18–36 (1 μM). Influx was measured after incubation at 37°C for 10 min. Each point is the mean ± S.D. of three separate experiments. A, ACh (□), ACh with NPY (◇), ACh with nifedipine (■), ACh with nifedipine and ω-Agatoxin IVA (△), ACh with nifedipine, ω-Agatoxin IVA and ω-conotoxin GVIA (▲) and ACh with NPY in the presence of nifedipine, ω-Agatoxin IVA and ω-conotoxin GVIA (◆). B, The legend is the same as in A except that the inclusion of NPY18–36 is denoted by (○) or (●). Each data point represents the mean ± S.D. of three separate experiments performed in triplicate.

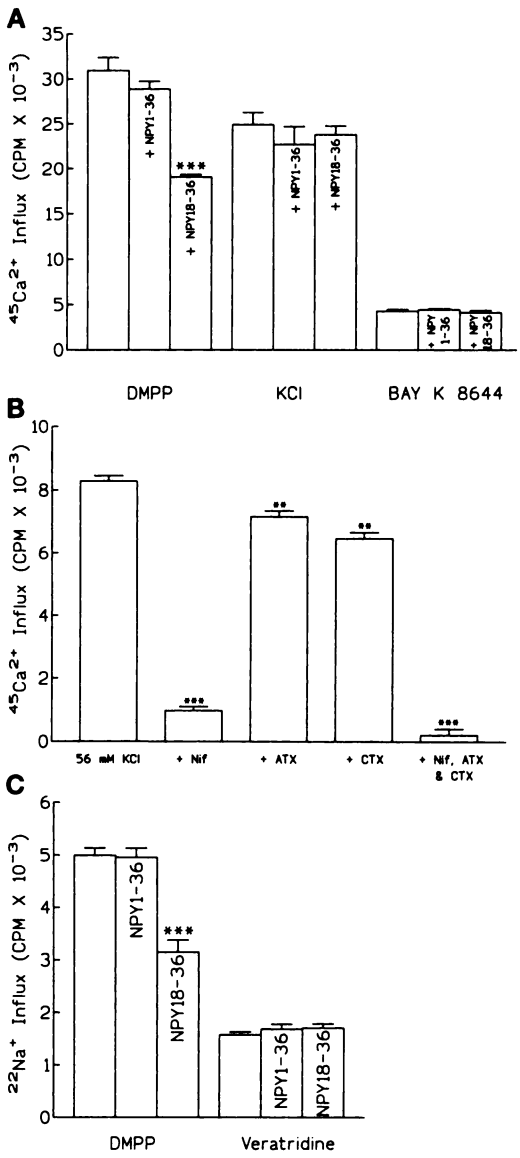

Fig. 3. NPY or NPY18–36 inhibition of $^{45}Ca^{++}$ or $^{22}Na^{+}$ influx induced by different mMethods of membrane depolarization. Chromaffin cells were stimulated with (A) DMPP (30 μM), KCl (56 mM), or BAY K 8644 (10 μM) or (C) with DMPP or veratridine (0.1 mM) in the absence or presence of NPY (1 μM) or NPY18–36 (1 μM) for 10 min at 37°C. B, 56 mM KCl was incubated with chromaffin cells in the presence of the voltage-gated calcium channel blockers (Nif, 30 μM; ATX, 0.1 μM; and CTX, 1 μM). Each data point represents the mean ± S.D. of three separate experiments performed in triplicate (basal influx subtracted). $^{**}P < .01$; $^{***}P < .001$.

duced $^{45}Ca^{++}$ influx by approximately 54%, 59% or 60%, respectively. However, the simultaneous addition of all three calcium channel blockers plus NPY18–36 resulted in the inhibition of ACh-induced $^{45}Ca^{++}$ influx by 88%.

Effect of NPY and NPY18–36 on $^{45}Ca^{++}$ or $^{22}Na^{+}$ influx increased by membrane depolarizing agents. NPY and NPY fragments are effective inhibitors of [^{3}H]NE secretion induced by nicotinic receptor stimulation but not by membrane depolarization using high K^{+} or veratridine (Hexum *et al.*, 1994). The effect of these peptides on increases in Na^{+} and Ca^{++} ion flux due to membrane depolarizing agents has not been examined. Stimulation of chromaffin cells by DMPP, KCl or BAY K 8644 resulted in varying increases in $^{45}Ca^{++}$ influx (fig. 3A). Inclusion of NPY during stimulation with these agents did not result in inhibition of $^{45}Ca^{++}$ influx. NPY18–36 inhibited the DMPP-induced increase in $^{45}Ca^{++}$ influx but not that produced by either KCl or BAY K 8644. We also compared various Ca^{++} channel antagonists for their ability to inhibit $^{45}Ca^{++}$ influx brought about by depolarizing concentrations of K^{+} (fig. 3B). The influx of $^{45}Ca^{++}$ was completely blocked by the simultaneous presence of the three types of Ca^{++} channel antagonists.

We then assessed the effect of NPY on $^{22}Na^{+}$ influx since nicotinic receptor-induced depolarization can activate voltage-gated Na^{+} channels. This could result in a subsequent increase in $^{45}Ca^{++}$ influx. The influx of $^{22}Na^{+}$ into chromaffin cells can be increased by both nicotinic receptor stimulation and the voltage-gated Na^{+} channel agonist veratridine (fig. 3C). NPY18–36 inhibited the DMPP-induced increase in $^{22}Na^{+}$ influx but not that produced by veratridine. The potencies of NPY and NPY fragments were compared for their ability to inhibit $^{22}Na^{+}$ influx into chromaffin cells (fig. 4). The rank order of potency was NPY18–36 ≥ NPY26–36 ≥

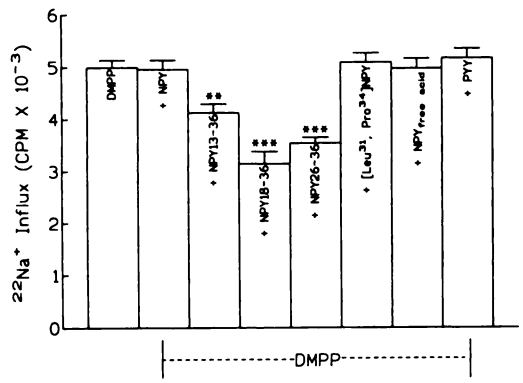

Fig. 4. Inhibition of DMPP-stimulated $^{22}Na^+$ influx by NPY or NPY fragments. Chromaffin cells were stimulated with DMPP (30 μM) or DMPP and NPY (1 μM) or NPY fragments (1 μM) at 37°C for 10 min. NPY or fragments did not alter basal $^{22}Na^+$ influx, which was 3267 ± 345 cpm. Each data point represents the mean ± S.D. of three separate experiments performed in triplicate (basal influx subtracted). **P < .01; ***P < .001.

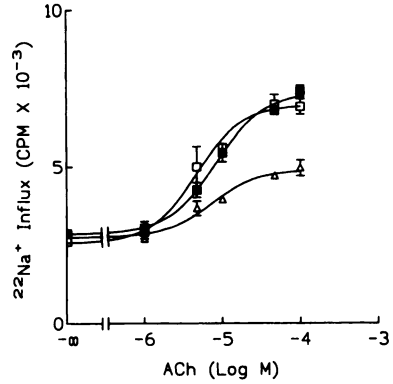

Fig. 6. Effect of tetrodotoxin on ACh-induced $^{22}Na^+$ influx. ACh (30 μM) [with atropine (0.5 μM) and physostigmine (10 μM)] was incubated with chromaffin cells with or without tetrodotoxin (1 μM) at 37°C for 10 min. Each data point represents the mean ± S.D. of three separate experiments performed in triplicate (basal influx subtracted). ACh with atropine (□), ACh with atropine and tetrodotoxin (■) and ACh with atropine, tetrodotoxin and NPY18–36 (1 μM) (△).

NPY13–36. NPY, [Leu^{31},Pro^{34}]NPY, NPY$_{free\ acid}$ and PYY were ineffective in altering $^{22}Na^+$ influx. Increasing concentrations of NPY18–36 gave a dose-response curve with an IC_{50} of 2.03×10^{-6} M (fig. 5).

Effect of NPY18–36 on voltage-gated sodium channels. The increase in $^{22}Na^+$ influx after veratridine treatment demonstrated the possible contribution of voltage-gated sodium channels to $^{22}Na^+$ influx after ACh-induced membrane depolarization. These channels, when activated, may amplify Ca^{++} influx into chromaffin cells by promoting opening of voltage-gated calcium channels. Therefore, one possible site through which NPY18–36 may decrease ACh-induced $^{45}Ca^{++}$ influx is the voltage-gated sodium channel. Chromaffin cells were treated with tetrodotoxin to block the voltage-gated sodium channels and subsequently depolarized by the addition of ACh. Addition of tetrodotoxin did not alter the ability of chromaffin cells to accumulate $^{22}Na^+$ after ACh stimulation (fig. 6), whereas tetrodotoxin completely blocked the veratridine-induced increase in $^{22}Na^+$ influx in chromaffin cells (data not shown). Moreover, the effect of NPY18–36 on ACh-induced $^{22}Na^+$ influx was still observed when voltage-gated sodium channels were blocked by tetrodotoxin.

Effect of NPY or NPY18–36 on ACh-induced [^{3}H]NE secretion in the presence of voltage-gated calcium channel blockers. The decrease in $^{45}Ca^{++}$ influx produced by calcium channel blockers, in the presence or absence of NPY, should be reflected in a similar decrease in [^{3}H]NE secretion from chromaffin cells. Nifedipine is an effective inhibitor of [^{3}H]NE secretion, whereas either ω-conotoxin GVIA or ω-agatoxin IVA inhibits secretion significantly although weakly (fig. 7). In combination, they can reduce ACh-induced [^{3}H]NE secretion by 61%. The addition of NPY produces a further although small decrease in [^{3}H]NE secretion. However, [^{3}H]NE secretion is reduced by 88% when all calcium channel blockers plus NPY18–36 are present (fig. 7).

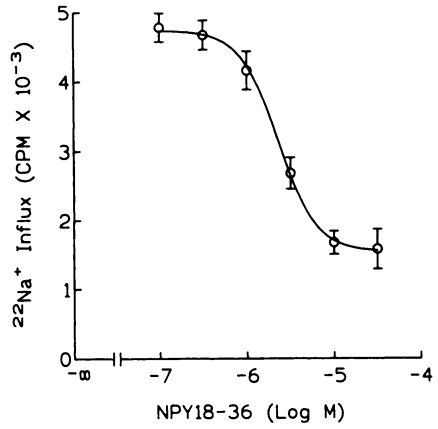

Fig. 5. Inhibition of DMPP-stimulated $^{22}Na^+$ influx by increasing concentrations of NPY18–36. Chromaffin cells were incubated (10 min at 37°C) with DMPP (30 μM) and various concentration of NPY18–36. Basal $^{22}Na^+$ influx was 3320 ± 425 cpm. Stimulated $^{22}Na^+$ influx by DMPP alone was 5133 ± 220 cpm (basal influx subtracted). Each data point represents the mean ± S.D. of three separate experiments performed in triplicate (basal influx subtracted).

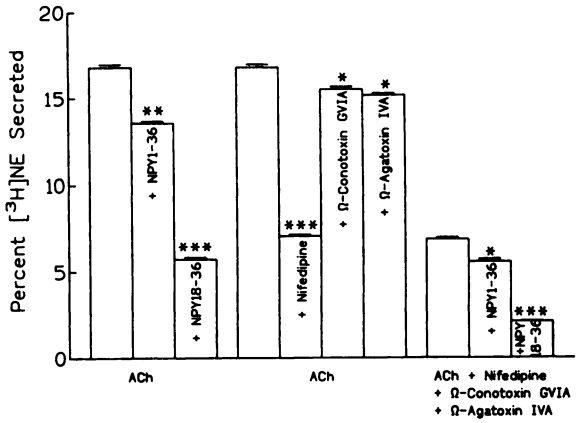

Fig. 7. Effect of NPY or NPY18–36 on ACh-induced [^{3}H]NE secretion. ACh (30 μM) [with atropine (0.5 μM) and physostigmine (10 μM)] was incubated with chromaffin cells without or with the voltage-gated calcium channel blockers nifedipine (50 μM), ω-Agatoxin IVA (0.4 μM) and ω-conotoxin (2 μM), in the absence or presence of NPY (1 μM) or NPY18–36 (1 μM), at 37°C for 10 min. [^{3}H]NE appearing in the culture was measured. Basal secretion of [^{3}H]NE, which was subtracted, was 4.755 ± 0.077. Each data point represents the mean ± S.D. of three separate experiments performed in triplicate. *P < .05, **P < .01; ***P < .001.

Correlation of NPY18–36 action on $^{45}Ca^{++}$ influx, $^{22}Na^{+}$ influx and [^{3}H]NE secretion. A plot of inhibition of ACh stimulated $^{45}Ca^{++}$ influx *vs.* inhibition of ACh-stimulated $^{22}Na^{+}$ influx at several concentrations of NPY18–36 showed a strong correlation between these two events (fig. 8A). An analysis by least-squares linear regression gave a slope of 2.04 and a correlation coefficient of .9697 that was significant at the $P < .0003$ level. Furthermore, a plot of percent inhibition of ACh-stimulated [^{3}H]NE secretion *vs.* inhibition of ACh-stimulated $^{22}Na^{+}$ influx at several concentrations of NPY18–36 showed a strong correlation between these two events (fig. 8B). An analysis by least-squares linear regression gave a slope of 3.45 and a correlation coefficient of .9894 that was significant at the $P < .0001$ level.

Effect of pertussis toxin treatment of chromaffin cells on NPY or NPY18–36 inhibition of DMPP-induced $^{45}Ca^{++}$ or $^{22}Na^{+}$ influx. Responses to NPY receptor stimulation may be mediated by G proteins (Michel, 1991), some of which are sensitive to the effects of pertussis toxin. Chromaffin cells were pretreated with pertussis toxin, and the effect of NPY18–36 on nicotinic receptor-stimulated $^{45}Ca^{++}$ and $^{22}Na^{+}$ influx was examined (fig. 9). We used the same conditions for pertussis toxin treatment that we previously demonstrated to effectively identify the involvement of a G protein in chromaffin cells function (Zhu *et al.*, 1992). NPY18–36 inhibited DMPP-induced $^{45}Ca^{++}$ influx to the same extent, either without (52%) or with (48%) pertussis toxin pretreatment (fig. 9A). Moreover, NPY18–36 inhibited DMPP-induced $^{22}Na^{+}$ influx by 52% in control cells and by 55% in cells pretreated with pertussis toxin (fig. 9B). NPY was an ineffective inhibitor of nicotinic receptor-stimulated ion flux in either case.

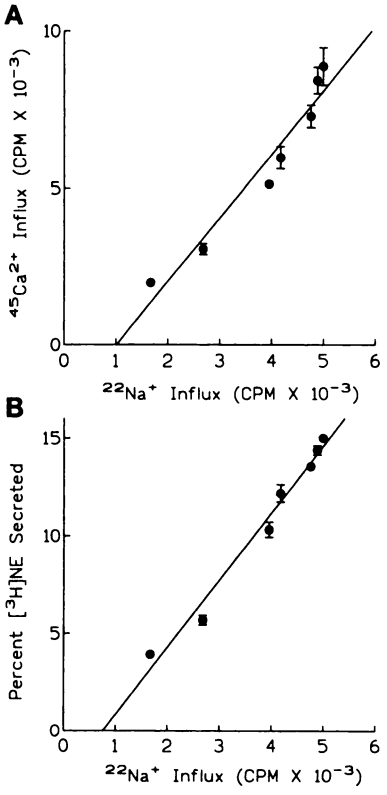

Fig. 8. Regression analysis of inhibition of $^{45}Ca^{++}$ influx and $^{22}Na^{+}$ (A) or [^{3}H]NE secretion and $^{22}Na^{+}$ (B) at several NPY18–36 concentrations. Chromaffin cells were incubated with DMPP (30 μM) and various concentrations of NPY18–36 at 37°C for 10 min. A, The 95% confidence limits for the regression line are shown as dotted curves ($P < .0003$, correlation coefficient = .9697). NPY18–36 did not alter either basal $^{45}Ca^{++}$ or $^{22}Na^{+}$ influx (4320 ± 192 cpm and 3345 ± 289 cpm, respectively), which was subtracted. B, The 95% confidence limits for the regression line are shown as dotted curves ($P < .0001$ correlation coefficient = .9894). NPY18–36 did not alter basal [^{3}H]NE secretion (4.656 ± 0.102), which was subtracted. Each data point represents the mean ± S.D. of three separate experiments performed in triplicate.

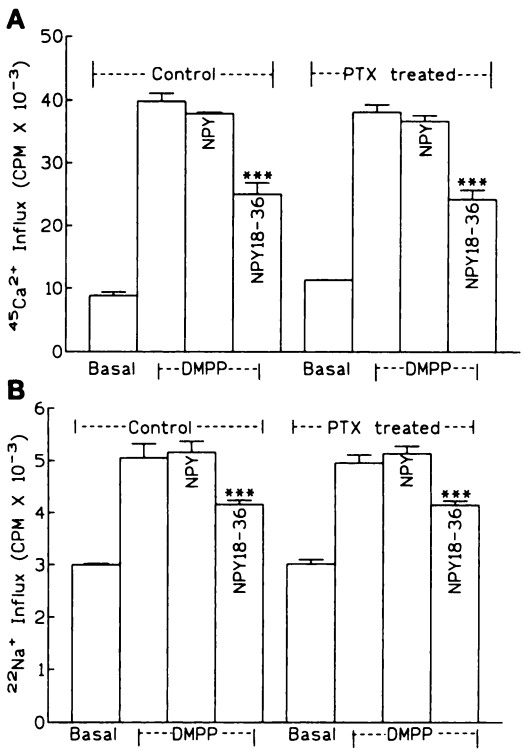

Fig. 9. Effect of pertussis toxin (PTX) treatment of chromaffin cells on NPY or NPY18–36 inhibition of $^{45}Ca^{++}$ (A) or $^{22}Na^{+}$ (B) influx induced by DMPP. Chromaffin cells were preincubated with buffer or PTX (0.1 μg/ml) for 18 hr at 37°C. Cells were then stimulated with DMPP (30 μM) or DMPP with NPY (1 μM) or NPY18–36 (1 μM) in 37°C for 10 min. Each data point represents the mean ± S.D. of three separate experiments performed in triplicate. ***$P < .001$.

TABLE 1

Comparison of IC_{50} values for inhibition of catecholamine secretion

Catecholamine	NPY	NPY18–36
Epinephrine	2.7×10^{-6} M	4.1×10^{-7} M
Norepinephrine	3.2×10^{-6} M	5.0×10^{-7} M

Effect of NPY and NPY18–36 on nicotine-induced secretion of epinephrine and NE from chromaffin cells. Chromaffin cells were stimulated with 3×10^{-5} M nicotine, and the secretion of epinephrine and NE was measured in the presence of increasing concentrations of either NPY or NPY18–36. NPY18–36 was a more effective inhibitor of secretion than NPY (table 1).

Discussion

Stimulation of chromaffin cell nicotinic receptors results primarily in an influx of Na^{+} through the nicotinic receptor ion channel (Wada *et al.*, 1984). The resulting membrane

depolarization can activate voltage-gated calcium and sodium channels. Calcium ion movement through voltage-gated calcium channels is fundamental to the increase in free intracellular Ca^{++} that is required for chromaffin cell catecholamine secretion. Increasing Na^+ influx through voltage-gated sodium channels results in propagation of the initial depolarization throughout the cell membrane, which may further promote Ca^{++} influx through voltage-gated calcium channels.

We have provided evidence that NPY and NPY fragments inhibit [^{3}H]NE secretion by modifying the cholinergic nicotinic receptor-mediated increase in $^{45}Ca^{++}$ influx (Hexum *et al.*, 1994). The effect of NPY18–36 on [^{3}H]NE secretion is only observed after nicotinic receptor-induced membrane depolarization and not after that due to the administration of either high K^+ concentrations or veratridine. This suggests that the effect of NPY18–36 is exerted at the level of the nicotinic receptor. The NPY receptor subtype facilitating this effect has been designated, Y_4, since the rank order of potency for the inhibition of secretion is NPY18–36 ≥ NPY26–36 > NPY13–36 > NPY ≥ $NPY_{free\ acid}$. This rank order of potency is not shared by any of the other previously characterized NPY receptor subtypes (Michel, 1991). We have examined the mechanism by which NPY inhibits [^{3}H]NE secretion in more detail, using NPY18–36 and a variety of agents with sites of action on either voltage-gated calcium or sodium channels.

Calcium ion gains entry into chromaffin cells through three voltage-gated calcium channels possessing different characteristics, designated L-, N- or P-type channels (Artalejo *et al.*, 1994). Ca^{++} influx through these channels can be blocked by selective agents such as nifedipine, ω-conotoxin GVIA and ω-agatoxin IVA, respectively. Each of these agents can reduce Ca^{++} influx and subsequently catecholamine secretion to the extent that each channel type is involved in the secretory process. Therefore, blockade of all of these channels might be expected to reduce cellular Ca^{++} uptake and, subsequently NE secretion, to negligible amounts if their activation is the only route for Ca^{++} entry into the cell.

The simultaneous addition of maximal concentrations of blocking agents for each voltage-gated calcium channel inhibits only 60% of the total $^{45}Ca^{++}$ influx due to nicotinic receptor stimulation. However, the inclusion of NPY18–36 with the three calcium channel blockers results in 88% inhibition of $^{45}Ca^{++}$ influx. Moreover, [^{3}H]NE secretion is similarly affected by the simultaneous presence of NPY18–36, nifedipine, ω-conotoxin GVIA and ω-agatoxin IVA, *i.e.*, secretion is inhibited by 88%. These data provide two important pieces of information. First, Ca^{++} influx in chromaffin cells can occur through means other than activation of L-, N- or P-type channels since blockade of these channels does not completely inhibit nicotinic receptor-induced $^{45}Ca^{++}$ influx. Second, NPY18–36 acts at a site distinct from the voltage-gated calcium channels since $^{45}Ca^{++}$ influx was decreased by an additional 30% by the addition of the peptide in the presence of the three channel blockers.

The most parsimonious explanation for these observations is that the additional site of Ca^{++} entry into chromaffin cells is through the nicotinic receptor ion channel rather than through voltage-gated calcium channels. It is well known that nicotinic channels display a Ca^{++} permeability approximately equivalent to AMPA subtypes of glutamate channels (Hille, 1992). Therefore, we propose that NPY18–36 inhibits [^{3}H]NE secretion by blocking Ca^{++} influx through the nicotinic receptor. Consistent with these data is the ineffectiveness of NPY18–36 on [^{3}H]NE secretion (Hexum *et al.*, 1994) and $^{45}Ca^{++}$ influx in response to membrane depolarization produced by high [K^+]. Furthermore, NPY18–36 is ineffective in reducing $^{45}Ca^{++}$ influx due to activation of L-type calcium channels using BAY K 8644. The presence of all three calcium channels blockers completely prevents $^{45}Ca^{++}$ influx in response to membrane depolarization with K^+. This provides strong evidence that these three calcium channels are the only voltage-gated channels involved in Ca^{++} influx in chromaffin cells. These observations lend additional support to the hypothesis that NPY18–36 inhibits chromaffin cell secretion by blocking ion flow through the nicotinic receptor.

If NPY18–36 inhibits secretion by blocking ion flow through the nicotinic receptor, then NPY18–36 should be effective in inhibiting $^{22}Na^+$ influx in response to nicotinic receptor stimulation. In contrast, NPY18–36 should not block $^{22}Na^+$ influx due to the addition of the voltage-gated sodium channel activator veratridine. As predicted, the addition of NPY18–36 reduces $^{22}Na^+$ influx after DMPP stimulation but not after the addition of veratridine. In addition, voltage-gated sodium channels probably play only a minor role since nicotinic receptor-induced increases in $^{22}Na^+$ influx are unaltered in the presence of tetrodotoxin compared with control. The rank order of potency for NPY and NPY fragments inhibition of $^{22}Na^+$ influx is the same as that for inhibition of [^{3}H]NE secretion and $^{45}Ca^{++}$ influx. Moreover, the IC_{50} for NPY18–36 for inhibition of $^{22}Na^+$ influx is the same as those for the inhibition of [^{3}H]NE secretion and $^{45}Ca^{++}$ influx. The NPY18–36 inhibition of $^{22}Na^+$ influx is highly correlated with the inhibition of $^{45}Ca^{++}$ influx, which is also highly correlated with inhibition of [^{3}H]NE secretion (Hexum *et al.*, 1994).

Two previous studies have addressed the role of NPY in the inhibition of nicotinic receptor-mediated catecholamine secretion from bovine chromaffin cells. Higuchi *et al.* (1988) suggested a modulatory role for NPY acting on a specific NPY receptor. They provided no insight into the mechanism other than to report that high concentrations of NPY (2×10^{-6} M) could inhibit release brought about by 56 mM KCl. The rank order of potency for inhibition of endogenous catecholamine secretion and displacement of N-propionyl[^{3}H]NPY from a high affinity binding site (human pancreatic polypeptide ≥ NPY >> PYY) is different from that reported here. They did not examine fragments of NPY. There are no obvious technical differences between this work and ours other than they measured endogenous catecholamine secretion. Our studies show that NPY18–36 is a more effective inhibitor of either endogenous catecholamine or [^{3}H]NE chromaffin cell secretion than NPY.

A more recent study using the whole-cell patch-clamp technique concluded that NPY and NPY16–36 are equally effective in inhibiting inward nicotinic currents (Noerenberg *et al.*, 1991). However, they reported the effect of these peptides to be completely abolished by the pretreatment of cells with pertussis toxin or including GDP[β-S] in the patch pipette solution. Their conclusion is obscured by the results of others that pertussis toxin can stimulate catecholamine secretion by

direct activation of L-type calcium channels (Ceña *et al.*, 1991). This action of pertussis toxin is independent of its well-characterized action on G proteins.

We conclude that the effect of NPY on catecholamine secretion is mediated by a previously uncharacterized NPY receptor subtype that preferentially recognizes NPY fragments over the intact molecule. This receptor subtype has been designated Y_4 and is associated with the nicotinic receptor. Occupation of this receptor results in blockade of ion movement through the nicotinic receptor ion channel decreasing Ca^{++} influx and, thus, catecholamine secretion. Structural modification of the peptide structure could result in the development of unusual nicotinic receptor antagonists.

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Send reprint requests to: Terry D. Hexum, Ph.D., Department of Pharmacology, University of Nebraska Medical Center, Omaha, NE 68198-6260.