

Inhibition of Acetylcholine Release from Guinea Pig Myenteric Neurons by Neuropeptide Y: GTP-Binding Protein Mediation¹

MICHAEL W. MULHOLLAND AND SALIM JAFFER

Department of Surgery, University of Michigan, Ann Arbor, Michigan 48109-0331

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Neuropeptide Y (NPY) is a unique peptide with wide distribution in central and peripheral nervous systems. In the guinea pig, NPY-positive fibers are prominent in the myenteric plexus. To test whether NPY inhibits myenteric plexus acetylcholine (ACh) release and to define mechanisms, a purified preparation of myenteric plexus neurons was derived from the teniae coli of neonatal guinea pigs and maintained in primary culture. Incubation of cultured neurons labeled with [³H]ACh in the presence of NPY (10⁻¹⁴-10⁻⁶ M) significantly inhibited basal ACh release (83 ± 16 to 58 ± 11% of control). NPY significantly inhibited ACh release stimulated by potassium (55 mM); by adenylate cyclase agonists forskolin (10⁻⁶ M) and cholera toxin (10⁻⁸ M); and by calcitonin gene-related peptide, cholecystokinin octapeptide, and vasoactive intestinal peptide (each 10⁻⁸ M). In each instance, the inhibitory effects of NPY were reversed by preincubation with pertussis toxin. Reversal of inhibitory effects by pertussis toxin suggests that the actions of NPY are mediated via an inhibitory GTP-binding protein. © 1991 Academic Press, Inc.

INTRODUCTION

Neuropeptide Y (NPY) is a unique 37 amino acid peptide that was first isolated from the porcine brain [1]. The peptide, with expression restricted to neurons, is widely distributed in both the central and peripheral nervous systems [2-4]. NPY has strong amino acid sequence homology with pancreatic polypeptide (PP) and peptide YY (PYY); the three compose a family of regulatory peptides [5, 6]. Pancreatic polypeptide immunoreactivity is found in endocrine cells of the pancreatic islets [7]. PYY immunoreactive cells have been localized within the mucosa of the gastrointestinal tract, especially the distal small intestine [8]. Within the digestive tract, NPY-positive neurons are found in the stomach, the intestine, and both the endocrine and exocrine portions of the pancreas [9, 10].

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In the guinea pig intestine, NPY-positive nerve fibers constitute approximately 5% of all neurons within the myenteric plexus (11). The gut contains both extrinsically and intrinsically derived NPY-positive neurons; extrinsic NPY nerve fibers have been reported to contain adrenergic neurotransmitters, the majority originating from postganglionic sympathetic neurons [12, 13]. However, denervation experiments in rats indicate the largest number of the NPY-positive fibers are intramural in origin [14]. Intrinsic NPY-immunoreactive nerve fibers are believed to be nonadrenergic [13]. Intrinsic NPY-positive myenteric plexus neurons also stain positively for vasoactive intestinal polypeptide and peptide histidine isoleucine (PHI) [15, 16]. This combination of anatomic findings suggests that NPY may have important actions on enteric neuronal activity.

Garzon and associates have reported that NPY reduces the resting tension of guinea pig longitudinal muscle strips [17]. NPY was also noted to reduce the excitatory effects of cholecystokinin octapeptide via a neurally mediated mechanism [17]. Close arterial infusion of NPY in the cat produces inhibition of colonic motility; the effect is not mediated by adrenergic receptors [18]. While the physiologic relevance of these actions of NPY within the myenteric plexus remains to be established, they suggest that the peptide may exert inhibitory actions upon enteric neurons.

The current investigation was performed to test the hypothesis that NPY inhibits release of acetylcholine from enteric neurons and to study the mechanism by which such an inhibitory action might be exerted. The studies were designed to directly test the ability of NPY to modulate acetylcholine release by using cultured myenteric plexus neurons. The involvement of neuronal adenylate cyclase in the activity of NPY was determined and the potential role of GTP-binding proteins in the observed effects was examined.

MATERIALS AND METHODS

Materials. The following chemicals were purchased: hemicholinium, collagenase, penicillin, streptomycin, veratridine, forskolin, 8-bromo cyclic AMP, cholera

toxin, pertussis toxin, poly-L-lysine (Sigma Chemical, St. Louis, MO), vasoactive intestinal peptide (VIP), substance P, calcitonin gene-related peptide (CGRP), cholecystokinin octapeptide (CCK-8) (Peninsula Labs, Belmont, CA), Medium 199, (GIBCO, Grand Island, NY), and Nu-Serum (Collaborative Research, Bedford, MA). Neonatal Duncan-Hartley guinea pigs were purchased from Simonsen Labs (Gilroy, CA). Tritiated choline and tritiated acetylcholine were obtained from New England Nuclear (Boston, MA).

Myenteric plexus neuronal culture. Primary culture of myenteric plexus neurons was accomplished by an adaptation of previously described methods [19, 20]. Neonatal Duncan-Hartley guinea pigs were sacrificed, and using aseptic technique, the longitudinal smooth muscle of the teniae coli was dissected from the cecum; care was exercised not to enter the lumen of the bowel. Teniae coli were divided into 5- to 6-mm segments and were transferred to a Petri dish containing Hanks' balanced salt solution (HBSS) with highly purified collagenase (1 mg/ml) and incubated at 37°C for 1.25–1.5 hr. Tissue fragments were then transferred to fresh HBSS where, under a dissecting microscope, loosely adherent smooth muscle cells were dissected from the myenteric plexus, producing a purified myenteric plexus preparation [21]. Cell viability was maintained up to 21 days in culture.

The tissues derived from each animal were divided into 12 segments (approximately 10^5 cells per segment). Isolated plexuses were explanted into tissue culture wells previously coated with poly-L-lysine (10 $\mu\text{g}/\text{ml}$). Plexuses were exposed to culture medium containing Medium 199 supplemented with nonessential amino acids, Na-pyruvate, Hepes buffer (1 M), and L-glutamate (200 mM) plus NU-Serum (10%), glucose (5 mg/ml), and penicillin-streptomycin solution (100 U/ml). Culture medium was exchanged every second day and antibiotics were omitted after the initial feeding. Acetylcholine release studies were performed after 5 to 7 days in culture. Cultured plexuses were used for study only if they were firmly adherent to the culture well and exhibited extensive outgrowth of dendritic processes. Each plexus culture was inspected for the presence of uniform neuronal growth.

Acetylcholine release studies. The release of [^3H]-labeled acetylcholine ([^3H]ACh) was studied after exposure of neurons to [^3H]choline (0.2 μM , specific activity 80 Ci/mmol) in oxygenated modified Krebs-Ringer buffer containing 50 μM physostigmine. Incubation with [^3H]choline was carried out at 37°C for 60 min in a Dubnoff metabolic incubator gassed with 95% O_2 -5% CO_2 . Tissues were then washed twice with fresh buffer and allowed to recover for 30 min before exposure to agonists.

Each neuronal aggregate was exposed to only one test condition. Plexuses were exposed to NPY for 30 min

before addition of agonists in those experiments utilizing coincubation with a test peptide. In experiments employing pertussis toxin, neurons were coincubated with the toxin for 18 hr prior to addition of agonists.

In all experiments, total ^3H was determined to indicate transmitter release. Labeled neurons were washed and then exposed to agonists in a release medium containing the following constituents: NaCl, 118 mM; KCl, 4.7 mM; NaHPO_4 , 1 mM; MgCl_2 , 0.5 mM; CaCl_2 , 2.5 mM; NaHCO_3 , 25 mM; glucose, 11.1 mM; physostigmine, 50 μM and hemicholinium, 10 μM . NaCl was adjusted to maintain osmolarity. Labeled neurons were exposed to agonists in this release medium for 15 min; the release medium was then aspirated and added to scintillation fluid. The remaining tissue was solubilized and then placed in scintillation fluid. Radioactivity was quantified by liquid scintillation spectrometry. [^3H]ACh was expressed as the percentage of total tissue ^3H released into the medium.

In order to verify that the ^3H released was ACh, column chromatography was used. After stimulation with the respective agonists, aliquots of release medium were applied to a cation exchange resin (Bio-Rex 70) to separate [^3H]ACh and [^3H]choline from other products. Unlabeled ACh and choline were applied to the column along with [^{14}C]ACh as a marker for ACh. The column was eluted with NaHPO_4 buffer and radioactivity was determined. Two peaks of radioactivity were observed and greater than 90% of the increase in ^3H occurring after stimulation was in the form of [^3H]ACh.

Data analysis. Fractional release was calculated by expressing the radioactivity in the release medium as the percentage of total radioactivity in the tissue at the time of the experiment. The percent release of [^3H]ACh over basal was calculated by dividing the fractional release produced by agonists by the spontaneous (basal) release. All results were expressed as group means \pm SEM. Each experiment was performed using the tissues derived from one animal; the sample size (n value) was taken as the number of guinea pigs; all experimental groups had n values of 6 to 8. Statistical analysis was performed using one-way analysis of variance. Significance was accepted as $P < 0.05$.

RESULTS

Effects of NPY on basal ACh release. To determine if NPY inhibits basal ACh release from cultured myenteric plexus neurons, we examined the effects of the peptide on [^3H]ACh release from unstimulated neuronal plexuses. Neurons were exposed to varying concentration of NPY for 15 min in release medium to which no agonists had been added. Mean 15-min basal release was $4.5 \pm 0.2\%$ in the absence of NPY. Basal release of [^3H]ACh was significantly suppressed ($68 \pm 10\%$ of control) by exposure of plexuses to NPY at concentrations

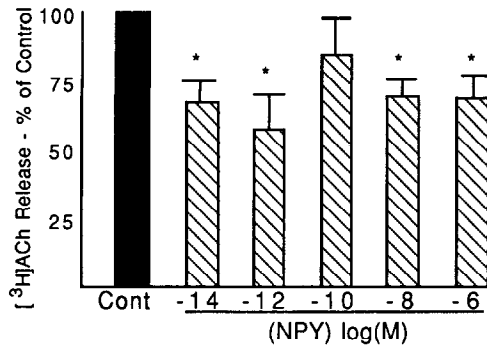


FIG. 1. Effect of NPY on basal [³H]ACh release from cultured myenteric plexus neurons. [³H]ACh-labeled neurons ($n = 6$ animals) were exposed to release medium in the absence or presence of graded concentrations of NPY for 15 min. [³H]ACh release was quantified as the percentage released into the medium relative to the total contained within the tissue at the beginning of the experiment. * $P < 0.05$ vs control.

as low as 10^{-14} M (Fig. 1). Statistically significant inhibitory effects were observed at each of the NPY concentrations employed except 10^{-10} M.

Effects of NPY on stimulated ACh release. Preliminary studies were performed to determine responsiveness of cultured neurons in terms of [³H]ACh release to various agonists. Exposure to potassium (55 mM) caused significant increases in [³H]ACh release ($261 \pm 47\%$ of basal). Dose-dependent increases in [³H]ACh release were noted with exposure to the depolarizing agent veratridine, with maximal stimulation noted at 10^{-6} M ($342 \pm 67\%$). Incubation with the cyclic AMP agonists forskolin, cholera toxin (CTX), or 8-bromo cyclic AMP was, in each instance, associated with dose-related increases in labeled ACh release. Exposure to peptide neurotransmitters was also associated with dose-related increases in [³H]ACh release. Incubation with CGRP or CCK-8 (10 nm) produced increases in [³H]ACh release of $73 \pm 33\%$ and $82 \pm 28\%$ above basal values, respectively. Substance P exposure dose-dependently increased [³H]ACh release ($179 \pm 62\%$ at 10 nm), as did VIP (10 nm incubation: $227 \pm 49\%$ of basal). Dose-response data were used to calculate ED_{50} values for each agonist. Submaximal agonist concentrations, for use in subsequent combination with NPY, were chosen based upon these agonist dose-response studies.

To investigate potential inhibitory effects of NPY on stimulated ACh release, we coincubated NPY with the above agonists. In experiments using NPY, the peptide (10^{-8} M) was added to the release medium 15 min before exposure to agonist. To investigate the possibility that NPY antagonizes stimulated ACh release via activation of inhibitory (G_i) regulatory proteins, in some experiments we exposed neurons to pertussis toxin (PTX) prior to addition of NPY and agonists. Pertussis toxin has been demonstrated to ribosylate the G_i protein and thereby to block the inhibitory actions of agents utiliz-

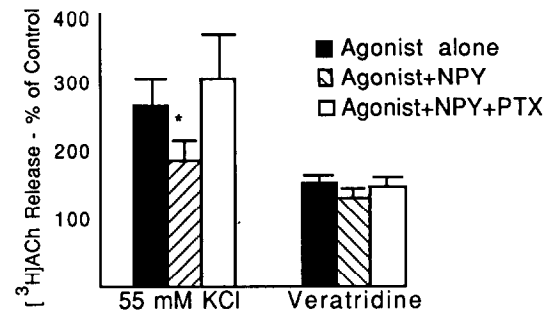


FIG. 2. Effects of NPY on potassium and veratridine-stimulated [³H]ACh release. Labeled neurons were exposed to potassium (55 mM) or to veratridine (10^{-8} M) in release medium. NPY (10^{-8} M) was added 15 min prior to exposure to agonists. In experiments utilizing PTX (100 ng/ml), toxin was added to culture medium 18 hr prior to exposure to agonists. * $P < 0.05$ vs control.

ing this mechanism. Pertussis toxin (100 ng/ml) was added 18 hr prior to [³H]choline exposure.

NPY produced significant suppression of potassium-stimulated [³H]ACh release ($190 \pm 36\%$ of basal) (Fig. 2). The inhibitory effects of NPY on potassium-stimulated [³H]ACh release were reversed by preincubation with PTX ($304 \pm 71\%$). Veratridine-stimulated (10^{-8} M) [³H]ACh was modestly decreased after exposure to NPY (from $144 \pm 5\%$ to $119 \pm 10\%$); the difference did not achieve statistical significance, however (Fig. 2).

[³H]ACh release stimulated by forskolin (10^{-6} M) was abolished by preincubation with NPY ($260 \pm 79\%$ vs $84 \pm 14\%$). The inhibitory effects of NPY were reversed by PTX pretreatment (Fig. 3). Similarly, NPY inhibited ACh release stimulated by CTX, decreasing fractional [³H]ACh release from $212 \pm 52\%$ to $72 \pm 10\%$ of basal. PTX pretreatment reversed NPY inhibition of CTX effects ($152 \pm 12\%$). In contrast to the effects observed with forskolin or CTX, stimulation of [³H]ACh release by 8-bromo cyclic AMP was not affected by NPY (Fig. 3).

In the last group of experiments, the ability of NPY to inhibit neuropeptide-stimulated ACh release was tested

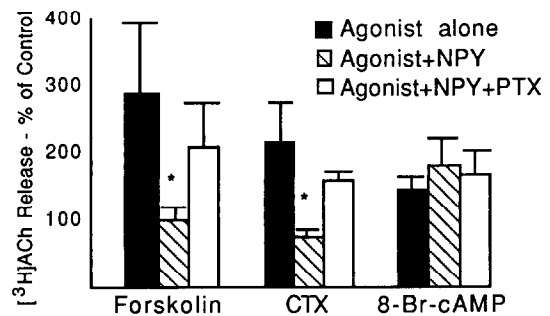


FIG. 3. Effects of NPY on [³H]ACh release stimulated by cAMP agonists. Labeled plexuses were stimulated by forskolin (10^{-6} M), cholera toxin (CTX: 10^{-8} M), or 8-bromo cyclic AMP (10^{-8} M). In accompanying wells, neurons were exposed to agonist plus NPY (10^{-8} M) or to NPY after preincubation with PTX. * $P < 0.05$ vs control.

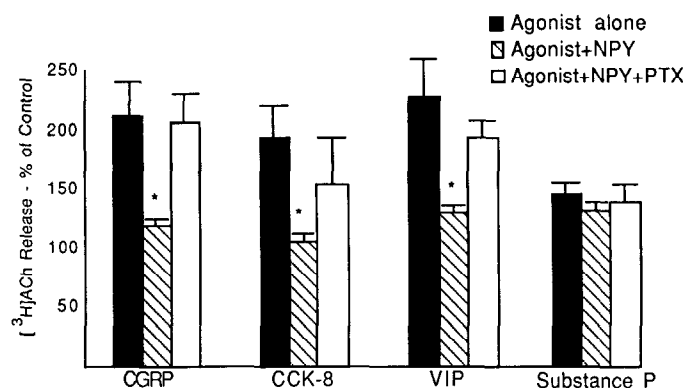


FIG. 4. Effects of NPY on neuropeptide-stimulated [^3H]ACh release. Cultured neurons were exposed to CGRP, CCK-8, VIP, or substance P (10^{-8} M) alone, or with NPY (10^{-8} M), or with NPY after PTX preincubation ($n = 8$). * $P < 0.05$ vs control.

(Fig. 4). NPY significantly inhibited CGRP-stimulated [^3H]ACh release ($209 \pm 31\%$ vs $120 \pm 9\%$); the inhibitory effects were reversed by PTX preexposure ($205 \pm 29\%$). CCK-8-stimulated ACh release ($192 \pm 28\%$ of basal) was significantly diminished by NPY coincubation ($110 \pm 13\%$); PTX preincubation reversed the inhibitory effects. Similarly, VIP stimulation of [^3H]ACh release was suppressed by NPY exposure ($227 \pm 49\%$ vs $130 \pm 12\%$), and the inhibitory effects were reversible with PTX pretreatment. In contrast, [^3H]ACh release stimulated by substance P was not inhibited by NPY coincubation.

DISCUSSION

This study has clearly demonstrated that NPY inhibits the release of ACh from cultured guinea pig myenteric plexus neurons. NPY was observed to suppress basal release of [^3H]ACh at concentrations as low as 10^{-14} M, indicating both its potent inhibitory effects and the sensitivity of the neurons in culture to respond to exogenous peptides. NPY was also noted to inhibit [^3H]ACh release stimulated by potassium-induced depolarization and by a variety of cholinergic secretagogues.

In preliminary studies, forskolin, cholera toxin, and 8-bromo cyclic AMP were each shown to stimulate [^3H]ACh release from myenteric plexus neurons, confirming previous reports that agents which raise intracellular cAMP levels stimulate the release of ACh from the myenteric plexus [22]. NPY was observed to inhibit the effects of forskolin, an agent which stimulates adenylate cyclase activity and the effects of CTX, an agent that activates adenylate cyclase indirectly through effects on stimulatory GTP-binding proteins [23, 24]. In contrast, [^3H]ACh release stimulated by 8-bromo cAMP, a soluble congener of cAMP, was not affected by NPY exposure. The ability of 8-bromo cAMP to mimic increased intracellular cAMP levels does not depend upon activation of adenylate cyclase. These observa-

tions, taken together, suggest that NPY inhibits cholinergic transmission in the myenteric plexus by inhibiting neuronal adenylate cyclase and preventing accumulation of intracellular cAMP. In support of this mechanism, NPY has recently been reported to inhibit forskolin-stimulated cAMP accumulation in the nucleus tractus solitarius region of the rat brain [25].

NPY was also demonstrated to inhibit ACh release stimulated by CGRP, CCK-8, or by VIP; the magnitude of the inhibitory effects were similar (approximately 45%) for each of the peptide agonists. Recent reports provide indirect evidence that intracellular cAMP accumulation may mediate ACh release from myenteric neurons caused by each of these peptides. CGRP has been reported to stimulate ACh release from myenteric plexus neurons. The release occurs via a mechanism that is sensitive to 2',5'-dideoxyadenosine [26]. Dideoxyadenosine has been shown to inhibit neuronal adenylate cyclase and to block the accumulation of intracellular cAMP. The ability of VIP to increase ACh release from myenteric plexus neurons is also sensitive to dideoxyadenosine, indicating activity via neuronal adenylate cyclase [27]. In addition, electrophysiological studies using myenteric plexus neurons have demonstrated that VIP and CCK-8 generate slow excitatory postsynaptic potentials, a pattern characteristically associated with cholinergic depolarization [28, 29]. The generation of slow excitatory postsynaptic potentials can be blocked by adenosine. The ability of NPY to inhibit [^3H]ACh release stimulated by CGRP, CCK-8, or VIP demonstrated in the current investigations is thus consistent with a mechanism involving inhibition of adenylate cyclase. In contrast, NPY was not noted to inhibit [^3H]ACh release stimulated by substance P. The effects of substance P are not mediated, in myenteric plexus neurons, via effects on intracellular cAMP.

The inhibitory actions of NPY on [^3H]ACh release were pertussis toxin sensitive. Guanine nucleotide-binding proteins have been demonstrated in a number of systems that couple inhibitory receptors to membrane-bound adenylate cyclase [30]. Such regulatory components (G_i) are sensitive to ADP-ribosylation catalyzed by pertussis toxin; exposure to pertussis toxin inactivates G_i and prevents inhibitory peptides from decreasing adenylate cyclase activity. The reversal of NPY inhibition of forskolin- and CTX-stimulated [^3H]ACh release by PTX pretreatment strongly suggests G protein involvement in the observed actions. PTX sensitivity of NPY inhibition of ACh release stimulated by CGRP, CCK-8, and VIP provides further support for the involvement of G_i protein in the actions of NPY in myenteric plexus neurons. Wiley and co-workers have demonstrated that an inhibitory guanine nucleotide-binding protein mediates NPY inhibition of ACh release from nodose ganglion neurons [31]. Additionally, in myenteric plexus neurons, inhibition of ACh release by somatostatin has also been shown to be PTX-sensitive

[22]. G protein mediation of adenylate cyclase activity, demonstrated in this study for NPY, may serve as a mechanism by which a variety of inhibitory neurotransmitters or hormones regulate cholinergic transmission within the myenteric plexus.

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