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Neuropeptide Y Enhances ATP-Induced Formation of Inositol Phosphates in Chromaffin Cells

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Bovine chromaffin cells contain high affinity NPY binding sites coupled through a pertussis toxin-sensitive G protein to inhibition of cAMP accumulation. NPY alone does not alter [^{3}H]inositol phosphate formation from [^{3}H]phosphoinositides in these cells. Increasing NPY concentrations, in the presence of ATP (300 μM), produced a dose-dependent enhancement in [^{3}H]-inositol phosphate formation, EC_{50} = 3.2 nM. Inclusion of the selective NPY-Y1 receptor antagonist BW1229 (1 μM) produced a marked decrease in NPY potency (EC_{50} = 3.3 μM). The Y1 receptor agonist, [Leu^{31}, Pro^{34}]-NPY, was equally effective with NPY, whereas NPY18-36, a Y2 receptor agonist, was much less effective. Inclusion of NPY with ATP also produced an enhancement in the release of intracellular Ca^{2+}. The ability of NPY to enhance both [^{3}H]inositol phosphate formation and the release of intracellular Ca^{2+} was pertussis toxin-insensitive. NPY action on bovine chromaffin cell receptor(s) appears to facilitated by different G proteins: one which can inhibit cAMP accumulation via a pertussis toxin-sensitive process and another which can enhance ATP activation of the inositol phosphate signaling pathway by a pertussis toxin-insensitive process. © 1997 Academic Press

Neuropeptide Y (NPY) is a thirty six amino acid peptide that is secreted from bovine chromaffin cells in response to nicotinic receptor stimulation (1). NPY exerts its effects primarily through inhibition of cyclic AMP (cAMP) formation or effects on Ca^{2+} influx (2). NPY has also been shown to have weak to moderate effects on phosphatidylinositol turnover (3). Chromaffin cells contain high affinity NPY binding sites, characteristic of the Y1 receptor subtype (4). These sites are coupled through a G protein, probably G_i, to the inhibition of forskolin-stimulated cAMP accumulation (4). However, NPY has no effect on $^{45}Ca^{2+}$ influx through chromaffin cell voltage-operated calcium channels in contrast to its effect in other tissues (5). The effect of NPY on inositol phosphate formation in chromaffin cells has not been reported.

Studies with other tissues have shown that NPY can increase inositol phosphate formation (6) or enhance the effects of other agents (7–9). Therefore, we examined whether NPY could increase inositol phosphate formation in chromaffin cells either alone or by enhancing the effects of another agent which increases inositol phosphate formation in these cells. ATP, which is released from bovine chromaffin cells in response to nicotinic receptor stimulation (10), can increase chromaffin cell inositol phosphate accumulation (11, 12). We present evidence demonstrating that although NPY alone cannot increase chromaffin cell inositol phosphate accumulation it enhances the ability of ATP to increase inositol phosphate accumulation. The effect is probably through the Y1 receptor subtype coupled to a pertussis-toxin-insensitive G protein. These data demonstrate the existence of another second messenger system utilized by NPY in chromaffin cells and support the contention that NPY and ATP may be comodulators of neurotransmission (13–15).

METHODS AND MATERIALS

Cell culture. Isolation and culturing of bovine adrenal chromaffin cells was performed as previously described (4). Cells were plated on 60 mm plastic dishes at a density of 4×10^6 cells/dish in an atmosphere of 5% CO_2 at 37°. When Ca^{2+} imaging experiments were performed, a poly-L-lysine coated coverslip was placed in the 60 mm plastic dish prior to plating the cells. The coverslip containing the cell was then removed and used. Cells were used between 3 and 8 days after plating.

Inositol phosphate determination. Assays were essentially as described previously (16, 17) with minor modifications. Cells grown on 35-mm dishes were labeled for 18 hr with 2 μCi [^{3}H]inositol in 1 ml inositol-free high glucose DMEM supplemented with 10% fetal bovine serum. After labeling, cells were rinsed once with DMEM-HEPES (DMEM, 20 mM HEPES, pH 7.4) and then stimulated for 20 min with various concentrations of agents in DMEM-HEPES containing 10 mM LiCl. Labeled compounds were then extracted from the cells with methanol, and chloroform and water were added as described (17). Inositol phosphates in the resulting aqueous phase were separated on Dowex 1-X8 (formate form) columns. Total inositol phosphates were eluted with 8 ml of 1 M ammonium formate and 0.1 M formic acid. Radioactivity in a 3-ml portion of the eluate (a) and a 0.375-ml portion of the organic phase containing the inositol

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phospholipids (b) were determined by liquid scintillation counting. The percent conversion of inositol phospholipids to inositol phosphates was calculated by the formula a/(a + b) × 100.

Ca^{2+} determination. Cells grown on glass cover slips were loaded with 7.1 μM fura 2-AM for 30 min at 37° in Ringer's solution of the following composition: 148 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1.6 mM Na_2HPO_4, 0.4 mM NaH_2PO_4, 1.5 mM $CaCl_2$, and 5 mM D-glucose. After loading, cells were washed twice and then incubated again for 20 min in Ringer's solution to allow for intracellular dye cleavage. The cover slips were inserted into the chamber and fura 2 was excited at wavelengths of 350 and 380 nm using a PTI Deltascan System as previously described (18). The values for Ca^{2+} were calculated as follows: $[Ca^{2+}] = K_d[(R - R_{min})/R_{max} - R)] \times (380_{min}/380_{max})$, where R_{min} and R_{max} are the fluorescence ratios in the absence (plus 3 mM EGTA) and presence of saturating Ca^{2+} (3 mM), respectively and K_d = 224 nM.

Materials. Collagenase, DNAse, ATP and general reagents were purchased from Sigma (St. Louis, MO). NPY was provided by Dr. Jean Rivier of The Salk Institute (La Jolla, CA). NPY fragments and related peptides were from Peninsula Laboratories, Inc. (Belmont, CA). [^{3}H]inositol was obtained from Amersham (Arlington Heights, IL). Fura 2-AM was obtained from Molecular Probes (Eugene, OR). Dulbecco's Modified Eagle Medium, fetal bovine serum, Hanks balanced salt solution and cell culture plasticware were from GIBCO (Gaithersburg, MD).

Statistics. Data were analyzed by One-way ANOVA followed by Tukey-Kramer Multiple Comparisons Test using GraphPad Instat. Curves were fit by nonlinear regression using GraphPad PRISM.

RESULTS AND DISCUSSION

Bovine chromaffin cells were kept in culture for a minimum of three days, loaded with [^{3}H]inositol and incubated with NPY. Neither NPY, NPY13-36, NPY18-36, nor [Leu^{31}, Pro^{34}]NPY (0.1 μM) produced any significant increase in [^{3}H]inositol phosphate formation above the baseline value (not shown). ATP (100 μM) provided a positive control by producing a 5-fold increase in [^{3}H]inositol phosphate formation (not shown). The addition of increasing amounts of ATP to the culture media resulted in a dose-dependent increase in [^{3}H]inositol phosphate formation, EC_{50} = 1.8 μM (Fig. 1). These data replicate those previously reported for bovine chromaffin cells (11). The inclusion of NPY with ATP in the culture media resulted in a two-fold enhancement in maximal [^{3}H]inositol phosphate formation with a decrease in ATP potency, EC_{50} = 12 μM. The effect of NPY was not apparent below 3 μM ATP and was maximal at 300 μM ATP.

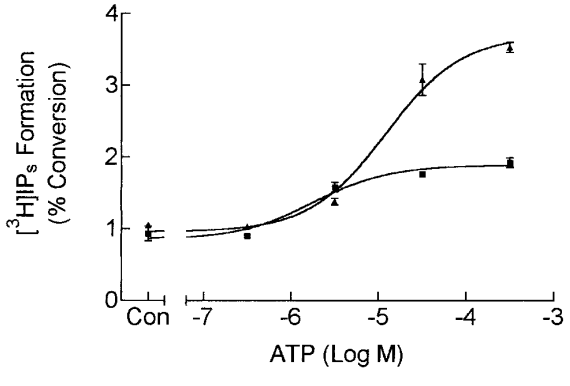

FIG. 1. NPY enhancement of ATP-stimulated [^{3}H]inositol phosphate formation. Bovine chromaffin cells were loaded with [^{3}H]-inositol (18 h) and incubated with increasing concentrations of ATP (■) or NPY (0.1 μM) plus ATP (▲) for 20 min at 37°C. Labeled compounds were extracted and separated, and the radioactivity was determined. The percent conversion of inositol phospholipids (b) to inositol phosphates (IPs) (a) was calculated using the formula a/(a + b) × 100. Each point is the average of triplicate determinations ± S.D. The data are representative of two experiments with similar results.

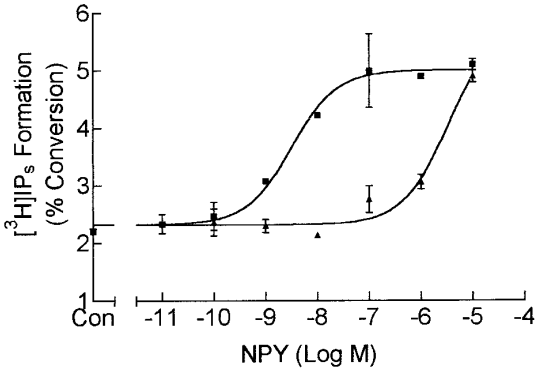

FIG. 2. Increasing NPY concentrations enhance ATP stimulation of [^{3}H]inositol phosphate formation. Chromaffin cells were loaded with [^{3}H]inositol and incubated with ATP (300 μM) and increasing concentrations of NPY (■) or NPY and the NPY-Y1 selective antagonist BW1229 (1 μM) (●) for 20 min at 37°C. Labeled compounds were extracted, separated and the radioactivity determined. The percent conversion of inositol phospholipids (b) to inositol phosphates (IPs) (a) was calculated using the formula a/(a + b) × 100. Each point is the average of triplicate determinations ± S.D. The data are representative of three experiments with similar results.

Increasing NPY concentrations, in the presence of ATP, produced a dose-dependent enhancement in [^{3}H]-inositol phosphate formation, EC_{50} = 3.2 nM (Fig. 2). Inclusion of the selective NPY-Y1 receptor antagonist BW1229 produced a 1000-fold decrease in NPY potency (EC_{50} = 3.3 μM) (Fig. 2). Similar results were obtained with the selective NPY-Y1 receptor antagonist, BIBP3226 (not shown). The NPY-Y1 receptor agonist [Leu^{31}, Pro^{34}]NPY was equally effective with NPY (Fig. 3) whereas NPY18-36 was much less effective (not shown). These data suggest that the ability of NPY to enhance ATP-stimulated inositol phosphate formation is mediated by the NPY-Y1 receptor.

Cells were pretreated with pertussis toxin to investigate whether either G_i or G_o plays a role in the action of ATP or NPY. Toxin-treated cells were examined for the ability of ATP and NPY to modulate [^{3}H]inositol phosphate formation. In untreated cells, ATP produced a 2.8-fold increase in [^{3}H]inositol phosphate (Fig. 4). Inclusion of NPY with ATP produced an additional 2.3-fold enhancement in [^{3}H]inositol phosphate formation. If cells were first treated for 24 hr with 100 ng/ml per-

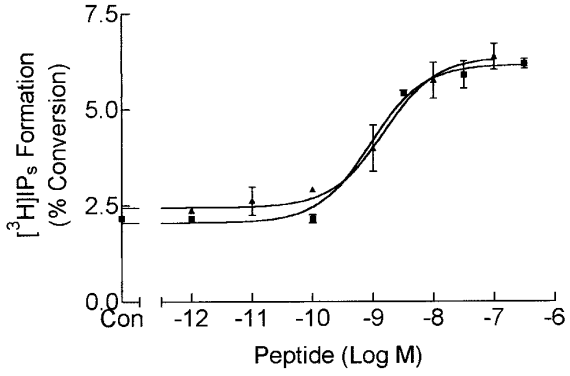

FIG. 3. Increasing [Leu[31], Pro[34]]NPY concentrations enhance ATP stimulation of [^{3}H]inositol phosphate formation. Chromaffin cells were loaded with [^{3}H]inositol and incubated with ATP (300 μM) and increasing concentrations of NPY (■) or [Leu[31], Pro[34]]NPY (▲) for 20 min at 37°C. Labeled compounds were extracted, separated and the radioactivity determined. The percent conversion of inositol phospholipids (b) to inositol phosphates (IPs) (a) was calculated using the formula a/(a + b) × 100. Each point is the average of triplicate determinations ± S.D. The data are representative of two experiments with similar results.

tussis toxin, ATP produced a 2.6-fold increase in [^{3}H]-inositol phosphate and inclusion of NPY with ATP produced an additional 1.9-fold enhancement in the [^{3}H]-inositol phosphate formation (Fig. 4). Thus the enhancing effect of NPY was only weakly inhibited by pertussis toxin treatment of cells. Values obtained from pertussis toxin-treated cells in the absence of ATP or ATP plus NPY were consistently lower than control due either to a weak effect of the toxin on [^{3}H]inositol

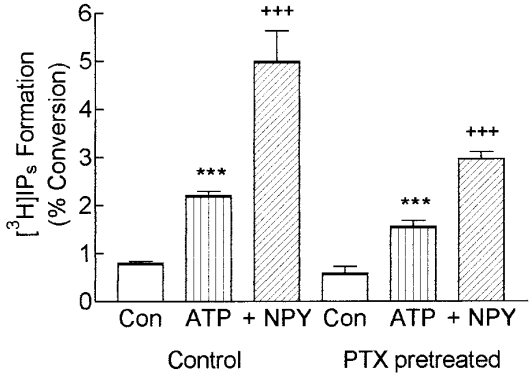

FIG. 4. Insensitivity of NPY enhancement of ATP stimulation of [^{3}H]inositol phosphate formation to pertussis toxin pretreatment. Chromaffin cells were loaded with [^{3}H]inositol (18 h) and incubated with ATP (300 μM) or ATP plus NPY (0.1 μM) for 20 min at 37°C. Pertussis toxin (100 ng/ml) treatment of cells was carried out during [^{3}H]inositol loading. Labeled compounds were extracted, separated and the radioactivity determined. The percent conversion of inositol phospholipids (b) to inositol phosphates (IPs) (a) was calculated using the formula a/(a + b) × 100. Each point is the average of triplicate determinations ± S.D. The data are representative of three experiments with similar results. ***, significantly different from (Con)trol ($p < 0.001$); $^{+++}$, significantly different from ATP alone ($p < 0.001$).

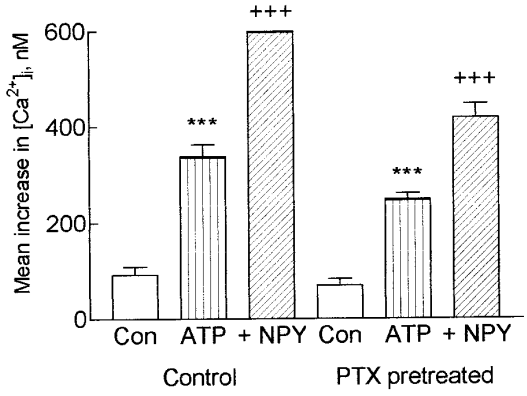

FIG. 5. Insensitivity of NPY enhancement of ATP stimulated increase in fura 2 fluorescence to pertussis toxin pretreatment. Chromaffin cells grown on glass cover slips were loaded with fura 2-AM and incubated with increasing concentrations of ATP (300 nM) or ATP plus NPY (0.1 μM) for 20 min at 37°C. Pertussis toxin treatment of cells was carried out at (100 ng/ml) for 18 h prior to fura 2-AM loading. Cells were excited at 350 and 380 nm. Intracellular Ca^{2+} was calculated as follows: $[Ca^{2+}] = K_d[(R - R_{min})/R_{max} - R)] \times (380_{min}/380_{max})$, where R_{min} and R_{max} are the fluorescence ratios in the absence (plus 3 mM EGTA) and presence of saturating Ca^{2+} (3 mM), respectively; K_d = 224 nM. Each point is the average of triplicate determinations ± S.D. The data are representative of three experiments with similar results. ***, significantly different from (Con)trol ($p < 0.001$); $^{+++}$, significantly different from ATP alone ($p < 0.001$).

loading or partial sensitivity of the endogenous agents responsible for control values to pertussis toxin pretreatment.

Cytoplasmic-free Ca^{2+} concentrations increase as a result of increased inositol phosphate formation (19). Accordingly, ATP produced a 3.7-fold increase in cytoplasmic-free Ca^{2+} as measured by increased fura 2 fluorescence (Fig. 5). Inclusion of NPY with ATP produced an additional 1.8-fold enhancement in cytoplasmic-free Ca^{2+}. If cells were first treated with pertussis toxin, ATP produced a 3.6-fold increase in cytoplasmic-free Ca^{2+} and inclusion of NPY with ATP produced an additional 1.7-fold enhancement (Fig. 5). This effect of NPY is therefore not pertussis toxin-sensitive either, similar to the NPY effect on ATP stimulated [^{3}H]inositol phosphate formation. There was a reduction in control values for pertussis toxin-treated cells similar to that seen when [^{3}H]inositol formation is being measured.

These data demonstrate that, although NPY alone has no effect on [^{3}H]inositol phosphate formation in bovine chromaffin cells, it can enhance [^{3}H]inositol phosphate production stimulated by ATP. The mechanism of the enhancing effect of NPY is unknown. We used pertussis toxin in an attempt to reveal the involvement of G_i or G_o as we have done previously (4). Our prior study demonstrated the effectiveness of pertussis toxin pretreatment as a tool to reveal the involvement of G_i or G_o in an NPY effect on chromaffin cells by completely eliminating the ability of NPY to inhibit forskolin-stimulated cAMP accumulation. The data

presented here show that these G proteins may be only partially involved since there is a small reduction in the NPY effect after pertussis toxin pretreatment. However, NPY in the presence of ATP produces a significant increase in [^{3}H]inositol phosphate formation in cells pretreated with pertussis toxin. This raises the possibility that NPY is coupled to a pertussis toxin-insensitive G protein such as G_q which increases inositol phosphate formation by activating phospholipase C (19). Thus, NPY may be coupled to two different G proteins in chromaffin cells.

Instances of neuropeptides exhibiting both pertussis toxin-sensitive and insensitive effects in the same cells have been previously demonstrated (6). Bradykinin receptors in rat dorsal root ganglion neurons can block Ca^{2+} currents via a pertussis toxin-sensitive process and stimulate phosphoinositide breakdown via a pertussis toxin-insensitive process. Thus NPY may be selectively linked to phospholipase C through a particular G protein distinct from that which inhibits cAMP accumulation. This effect of NPY is somehow dependent upon the prior action of ATP which may be to increase the amount of cytoplasmic-free Ca^{2+}.

Chromaffin cell NPY receptors have been characterized as NPY-Y1 and shown to be coupled to G_i (4). These data suggest that the NPY-Y1 receptor also couples to a pertussis toxin-insensitive G protein. The data reported here suggests that NPY enhances inositol phosphate formation through an effect on a NPY-Y1 receptor subtype. This conclusion derives from the observations that: a) the NPY-Y1 selective NPY antagonists BW1229 and BIBP3226, antagonize NPY action and b) the NPY-Y1 selective agonist [Leu^{31}, Pro^{34}]NPY but not the Y2 preferring agonist NPY18-36 can mimic the enhancing effect of NPY. ATP and NPY may be co-modulators of neurotransmission in chromaffin cells. These results are similar to those reported for the enhancing effects of NPY on ATP-induced contraction of the rat superior mesenteric artery (15).

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