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BIBP 3226 inhibition of nicotinic receptor mediated chromaffin cell secretion

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Abstract

(*R*)-*N*^{2}-(diphenacetyl)-*N*-[(4-hydroxyphenyl)methyl]-argininamide (BIBP 3226) is a selective neuropeptide Y Y_1 receptor antagonist with structural similarity to the C-terminal tripeptide of neuropeptide Y. Based on this similarity we questioned whether BIBP 3226 could act as an agonist. Incubation of BIBP 3226 with bovine chromaffin cells in culture results in the inhibition of nicotinic receptor-stimulated catecholamine secretion ($IC_{50} = 2.4$ μM). The effect of BIBP 3226 is independent of neuropeptide Y action since the presence of neuropeptide Y in the culture medium does not alter the effect of BIBP 3226. BIBP 3226 decreased the efficacy of the nicotinic receptor agonist, 1,1-dimethyl-4-phenylpiperizinium (DMPP), but did not change its potency suggesting non-competitive inhibition. BIBP 3226 has a similar effect on nicotinic receptor-stimulated $^{45}Ca^{2+}$ influx. BIBP 3226 does not inhibit [^{3}H]norepinephrine release induced by high K^+ and its effect is not pertussis toxin-sensitive. We conclude that not only can BIBP 3226 act as a neuropeptide Y receptor antagonist in bovine chromaffin cells but also act as an agonist and inhibit catecholamine secretion.

Keywords: Neuropeptide Y; BIBP 3226; Catecholamine secretion; Chromaffin cell

1. Introduction

BIBP 3226 is a selective neuropeptide Y Y_1 receptor antagonist (Rudolf et al., 1994) that acts in a competitive fashion (Doods et al., 1996). It was developed from the hypothesis that the neuropeptide Y pharmacophoric groups that interact with the neuropeptide Y Y_1 receptor are located in the C-terminus portion of the peptide (Beck et al., 1990). Thus modifications of the C-terminal tripeptide of neuropeptide Y, Gln–Arg–Tyr–NH_2, resulted in the development of BIBP 3226 ((*R*)-*N*^{2}-(diphenacetyl)-*N*-[(4-hydroxyphenyl)methyl]-argininamide) (Rudolf et al., 1994). BIBP 3226 has been shown to antagonize the effect of neuropeptide Y in various tissues including bovine adrenal chromaffin cells. BIBP 3226 antagonizes the effect of neuropeptide Y on inositol phosphate (Zheng et al., 1997) and cyclic AMP accumulation in chromaffin cells (Zheng, J. and Hexum, T.D., unpublished).

We have recently demonstrated that neuropeptide Y inhibits [^{3}H]norepinephrine nicotinic receptor-stimulated secretion from bovine adrenal chromaffin cells by blocking $^{45}Ca^{2+}$ influx through the nicotinic receptor ligand-gated ion channel. Neuropeptide Y-(18–36) and shorter neuropeptide Y fragments are more effective than neuropeptide Y in inhibiting secretion or $^{45}Ca^{2+}$ influx (Hexum et al., 1994) (Zheng et al., 1995). BIBP 3226 could not antagonize the effect of either neuropeptide Y or neuropeptide Y fragments on [^{3}H]norepinephrine secretion (Zheng, J. and Hexum, T.D., unpublished). In fact, nicotinic receptor-stimulated secretion was suppressed in the presence of BIBP 3226 alone. Since BIBP 3226 is a derivative of the C-terminus of neuropeptide Y we reasoned that BIBP 3226 may have an action similar to that of neuropeptide Y fragments. The results presented here examine its effect on [^{3}H]norepinephrine secretion and $^{45}Ca^{2+}$ influx and show that BIBP 3226 inhibits nicotinic receptor-stimulated [^{3}H]norepinephrine secretion and $^{45}Ca^{2+}$ influx in bovine chromaffin cells.

2. Materials and methods

2.1. Cell culture

The isolation and culture of bovine adrenal chromaffin cells was performed with modifications as previously described (Zhu et al., 1992). The cells were plated in 24-well

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plastic culture plates at a density of 1×10^6 cells/well in an atmosphere of 5% CO_2 at 37°C. One-half of the medium was changed every other day. Chromaffin cells used in studies were kept in culture for 3–7 days.

2.2. Secretion of norepinephrine and epinephrine

Secretion was studied by measuring the release of endogenous catecholamines. Culture media was removed from chromaffin cells by two washes with Krebs–Ringer phosphate (KRP) buffer, pH 7.4, containing 154 mM NaCl, 2.2 mM $CaCl_2$, 5.6 mM KCl, 1.1 mM $MgSO_4$, 0.85 mM NaH_2PO_4, 2.15 mM Na_2HPO_4 and 10 mM glucose. Chromaffin cells were then incubated in KRP at 37° for 15 min and stimulated with acetylcholine (3 μM) for 12 min at 37°. Media were removed and stabilized with 1.6 mM $NaHSO_3$ and 34 mM $HClO_4$ and stored at −20°C. Cell catecholamine content was determined on the lysate obtained after freeze-thawing cells in KRP buffer containing 0.1 mM EDTA and 0.1% Triton X-100 followed by stabilization with 1.6 mM $NaHSO_3$ and 34 mM $HClO_4$. Epinephrine and norepinephrine were measured by electrochemical detection after liquid chromatography (Hexum and Russett, 1989).

2.3. Secretion of [^{3}H]norepinephrine

Secretion was studied by measuring the release of [^{3}H]norepinephrine from chromaffin cells. The culture media was removed from the chromaffin cells followed by two washes with KRH, which contained 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. We added 0.5 μC_i of [^{3}H]norepinephrine to each well and incubated the cells for 90 min at 37°C in an atmosphere of 5% CO_2. The cells were washed three times followed by incubation at 37°C for 10 min in KRH. Secretion resulted after the addition of stimulating agents dissolved in KRH and incubation for 10 min at 37°C. The effects of BIBP 3226 or others were examined by a preincubation of the cells with the selected chemicals 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 of 1 N NaOH. The radioactivity was determined by liquid scintillation spectrometry. Secretion of [^{3}H]norepinephrine is expressed as the percentage of counts per minute in the incubation medium relative to the total counts per minute (medium plus cellular).

2.4. $^{45}Ca^{2+}$ influx

Chromaffin cells were washed twice with KRH (Ca^{2+}-free) (the same KRH as above except no $CaCl_2$). The cells were incubated (37°C) in KRH (Ca^{2+}-free) for 60 min. Calcium uptake was initiated by the addition of 0.5 ml KRH (Ca^{2+}-free), which contained 0.5 μC_i of $^{45}Ca^{2+}$ and either stimulating agents or stimulating agents plus BIBP 3226. Cells were preincubated with BIBP 3226 for 10 min at 37°C prior to stimulation. The reaction was terminated by withdrawing the stimulating solution and adding 0.5 ml of KRH (Ca^{2+}-free) plus 10 mM EGTA followed by three washes with this buffer. The cells were solubilized with 0.4 ml NaOH (1 N) followed by neutralization with 0.1 ml of HCl (4 N). The radioactivity was measured by scintillation spectroscopy.

2.5. Data analysis

Data were analyzed by One-way analysis of variance followed by the Tukey–Kramer Multiple Comparisons Test using GraphPad Instat™. Curves were fit and analyzed using GraphPad Prism™.

Table 1
Effect of BIBP 3226 on nicotinic receptor-stimulated catecholamine secretion and synthesis

Condition	Norepinephrine (nmoles/10^6 cells)	Epinephrine (nmoles/10^6 cells)
Control[c]	0.6 ± 0.03	0.9 ± 0.03
Acetylcholine[c]	2.2 ± 0.2^{aa}	3.7 ± 0.3^{aa}
Acetylcholine + hexamethonium[c]	0.8 ± 0.03^{bbb}	1.1 ± 0.1^{bbb}
Acetylcholine + neuropeptide Y[c]	2.0 ± 0.2^{aa}	3.2 ± 0.3^{aa}
Acetylcholine + BIBP 3226	1.2 ± 0.08^{bbb}	1.7 ± 0.2^{bbb}
Acetylcholine + BIBP 3226 + neuropeptide Y	1.4 ± 0.1^{bb}	1.8 ± 0.02^{bbb}

Chromaffin cells were stimulated with acetylcholine (3 μM) for 12 min at 37° and secretion of norepinephrine and epinephrine was measured by electrochemical detection after liquid chromatography. Hexamethonium (300 μM), neuropeptide Y (0.01 μM) and BIBP 3226 (0.3 μM) were added with acetylcholine. Neither hexamethonium, neuropeptide Y nor BIBP 3226 had any effect on secretion occurring in the absence of acetylcholine. Secretion of norepinephrine and epinephrine was 12.0% and 14.6%, respectively, of the cellular content of either catecholamine prior to stimulation with acetylcholine. Each point is the average of triplicate determinations ± S.E.M.; the data are representative of three individual experiments with similar results. Significance was determined by the Tukey–Kramer Multiple Comparisons Test. [aa]Significantly different from control ($p < 0.001$); [bb]Significantly different from acetylcholine alone ($p < 0.01$); [bbb]Significantly different from acetylcholine alone ($p < 0.001$).
[c]Data taken from Zheng et al. (1997).

3. Results

3.1. Inhibition of acetylcholine-induced catecholamine secretion by BIBP 3226

The addition of acetylcholine to chromaffin cell culture media results in the secretion of norepinephrine and epinephrine (Table 1). The secretion of either catecholamine was blocked by the prior addition of hexamethonium. The inclusion of neuropeptide Y at a concentration (0.01 μM) that activates a receptor-mediated event in chromaffin cells (Zheng et al., 1997) with acetylcholine did not alter the effect of acetylcholine on catecholamine secretion. However, the inclusion of BIBP 3226 (0.3 μM, a concentration that antagonizes this effect of neuropeptide Y) with acetylcholine, decreased secretion of norepinephrine by 45% and epinephrine by 54%. Moreover, the combined effect of neuropeptide Y and BIBP 3226 plus acetylcholine was no different from that of BIBP 3226 plus acetylcholine.

3.2. Inhibition of DMPP-induced [^{3}H]norepinephrine secretion by BIBP 3226

Increasing concentrations of BIBP 3226 (0.1–10 μM) produced a concentration-dependent inhibition of DMPP-stimulated [^{3}H]norepinephrine secretion, $IC_{50} = 2.4$ μM (Fig. 1). Incubation of chromaffin cells with BIBP 3226 alone did not alter basal [^{3}H]norepinephrine secretion. The inclusion of BIBP 3226 (3 μM) with increasing 1,1-dimethyl-4-phenylpiperizinium (DMPP) concentrations decreased the efficacy of DMPP but did not influence potency: DMPP—$IC_{50} = 5.3$ μM; DMPP plus BIBP 3226—$IC_{50} = 6.9$ μM (Fig. 2). Chromaffin cell secretion can also be stimulated by membrane depolarization. BIBP 3226 had no effect on [^{3}H]norepinephrine secretion induced by 56 mM KCl (data not shown). Another neuropeptide Y Y_1 receptor antagonist, BW 1229, (Daniels et al., 1995) had a

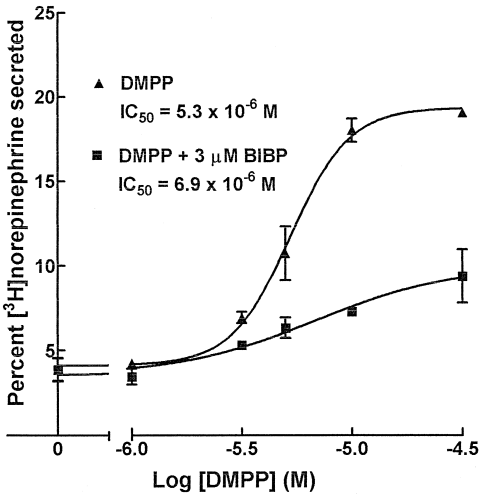

Fig. 2. BIBP 3226 inhibition of [^{3}H]norepinephrine secretion with increasing DMPP concentrations. Chromaffin cells were stimulated with increasing DMPP concentrations in the presence or absence of BIBP 3226 (3 μM) for 10 min at 37°C. Each data point represents the mean ± S.D. of triplicate determinations. The data is representative of three separate experiments with similar results.

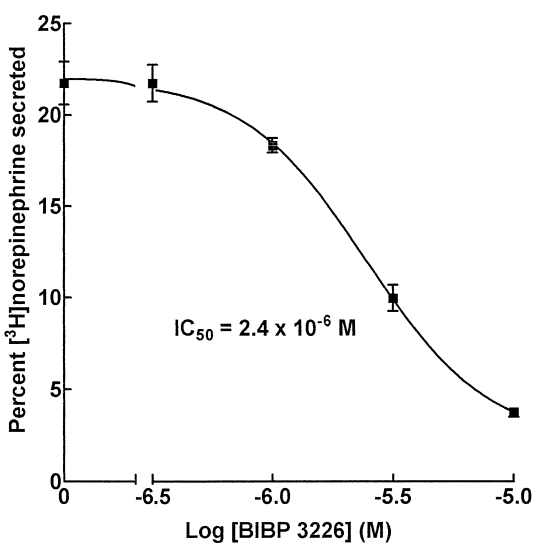

Fig. 1. Inhibition of DMPP-stimulated [^{3}H]norepinephrine secretion by increasing BIBP 3226 concentrations. Bovine chromaffin cells were stimulated with DMPP (30 μM) or DMPP in the presence of different BIBP 3226 concentrations for 10 min at 37°C. BIBP 3226 did not alter basal [^{3}H]norepinephrine secretion. Each data point represents the mean ± S.D. of triplicate determinations. The data is representative of three separate experiments with similar results.

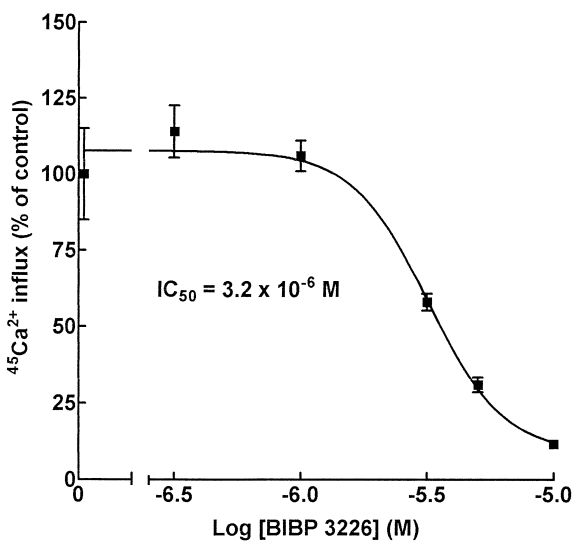

Fig. 3. Inhibition of DMPP-stimulated $^{45}Ca^{2+}$ influx by increasing BIBP 3226 concentrations. Chromaffin cells were stimulated with DMPP (30 μM) or DMPP in the presence of different BIBP 3226 concentrations for 10 min at 37°C. BIBP 3226 did not alter basal $^{45}Ca^{2+}$ influx which was 13055 cpm ± 60/10^6 cells. Each data point represents the mean ± S.D. of triplicate determinations. The data is representative of three separate experiments with similar results.

similar effect on [^{3}H]norepinephrine secretion (data not shown).

The effect of neuropeptide Y on chromaffin cell cyclic AMP accumulation is mediated by a pertussis toxin-sensitive G-protein (Zhu et al., 1992). However, pertussis toxin treatment of chromaffin cells did not alter the effect of either BIBP 3226 (data not shown) or neuropeptide Y (Zheng et al., 1995) on [^{3}H]norepinephrine secretion.

3.3. Effect of BIBP 3226 on $^{45}Ca^{2+}$ influx

Ca^{2+} is an obligatory requirement for chromaffin cell secretion. DMPP stimulation of chromaffin cells increases $^{45}Ca^{2+}$ influx (Zheng et al., 1995) and initiates [^{3}H]norepinephrine secretion (Hexum et al., 1994). BIBP 3226 dose dependently inhibited DMPP-induced $^{45}Ca^{2+}$ influx ($IC_{50} = 3.2$ μM) (Fig. 3). Incubation of chromaffin cells with BIBP 3226 alone did not alter basal $^{45}Ca^{2+}$ influx. The inclusion of BIBP 3226 (3 μM) with increasing DMPP concentrations decreased the efficacy of DMPP but did not influence potency: DMPP: $IC_{50} = 2.1$ μM; DMPP plus BIBP 3226: $IC_{50} = 3.4$ μM (Fig. 4). Stimulation of $^{45}Ca^{2+}$ influx by addition of K^+ (56 mM) was not affected by BIBP 3226 (not shown).

3.4. Correlation of BIBP 3226 action on $^{45}Ca^{2+}$ influx and [^{3}H]norepinephrine secretion

A plot of DMPP-stimulated $^{45}Ca^{2+}$ influx vs. DMPP-stimulated [^{3}H]norepinephrine secretion at several concentrations of BIBP 3226 showed a strong correlation between these two events (Fig. 5). Linear regression analysis gave a slope of 5.8 that was significantly different from zero at the $P < 0.01$ level and a coefficient of determination (r^2) of 0.98.

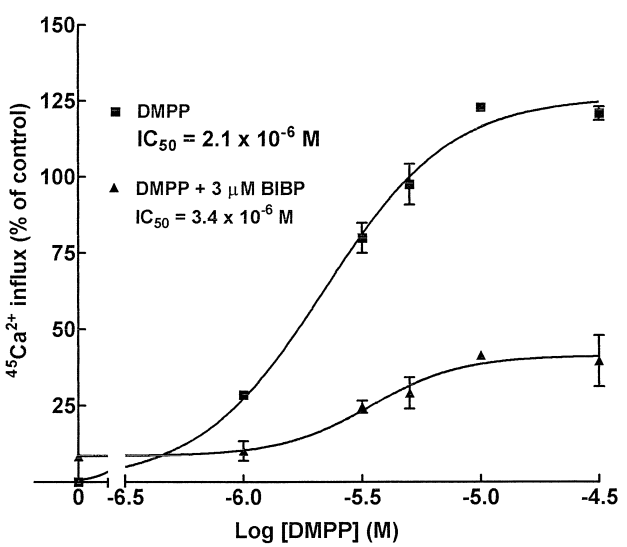

Fig. 4. BIBP 3226 inhibition of $^{45}Ca^{2+}$ influx with increasing DMPP concentrations. Chromaffin cells were stimulated with increasing DMPP concentrations in the presence or absence of BIBP 3226 (3 μM) for 10 min at 37°C. Each data point represents the mean ± S.D. of triplicate determinations. The data is representative of three separate experiments with similar results.

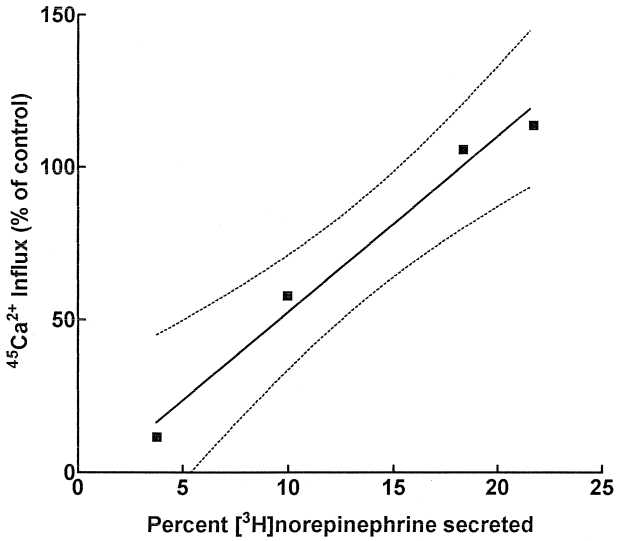

Fig. 5. Regression analysis of BIBP 3226 inhibition of $^{45}Ca^{2+}$ influx and [^{3}H]norepinephrine secretion. Data from Fig. 1Fig. 3 were plotted and analyzed by linear regression. The 95% confidence limits for the regression line are shown as dotted curves ($P < 0.01$, coefficient of determination (r^2) = 0.98.

4. Discussion

Nicotinic receptor stimulation of bovine chromaffin cells using acetylcholine results in the secretion of epinephrine and norepinephrine: 12.0% and 14.6%, respectively, of the total cell content of epinephrine or norepinephrine before secretion. The inclusion of neuropeptide Y (0.01 μM) in the culture medium does not inhibit secretion in contrast to data published previously (Higuchi et al., 1988). The current data are in agreement with our previous findings (Hexum et al., 1994; Zheng et al., 1995), however, where we showed that high concentrations (μM) of neuropeptide Y and the more potent fragment, neuropeptide Y-(18–36), are required to inhibit [^{3}H]norepinephrine secretion. These peptides do not act through a typical neuropeptide Y receptor but through an effect on the nicotinic receptor ligand-gated ion channel.

BIBP 3226 is a selective neuropeptide Y Y_1 receptor antagonist. It is structurally related to the C-terminal tripeptide of neuropeptide Y. Since the C-terminal fragments of neuropeptide Y can inhibit [^{3}H]norepinephrine secretion we decided to examine the effect of BIBP 3226 on nicotinic receptor stimulated chromaffin cell secretion. First we studied its effect in the presence and absence of neuropeptide Y on secretion of endogenous catecholamines from chromaffin cells. Neuropeptide Y had no effect on secretion while BIBP 3226 (0.3 μM) inhibited

norepinephrine and epinephrine secretion by 45 and 54%, respectively. The combination of neuropeptide Y and BIBP 3226 was no different from that of BIBP 3226 alone.

Next we examined the effect of increasing BIBP 3226 concentrations on DMPP stimulated secretion from chromaffin cells loaded with [^{3}H]norepinephrine. DMPP is a nicotinic receptor ligand with selectivity for the neuronal receptor subtype. BIBP 3226 is an effective inhibitor of [^{3}H]norepinephrine secretion, $IC_{50} = 2.4$ μM. BIBP 3226, in the presence of increasing DMPP concentrations did not effect the potency of DMPP as a secretogogue but decreased its efficacy, suggesting non-competitive inhibition by BIBP 3226.

Another set of experiments where cells were loaded with $^{45}Ca^{2+}$ gave similar results. The IC_{50} for BIBP 3226 inhibition of IC_{50} influx is 3.2 μM which compares favorably with the inhibition of [^{3}H]norepinephrine secretion by BIBP 3226 (2.4 μM). Similarly, BIBP 3226 did not affect the potency of DMPP to increase $^{45}Ca^{2+}$ influx but did decrease the efficacy. Regression analysis revealed that the effect of BIBP 3226 on [^{3}H]norepinephrine secretion and $^{45}Ca^{2+}$ influx are highly correlated, $r^2 = 0.98$.

We also examined the effect of BIBP 3226 on K^+-induced secretion of [^{3}H]norepinephrine as well as $^{45}Ca^{2+}$ influx and found no effect. This suggests that BIBP 3226 does not exert its effect on voltage-operated Ca^{2+} channels. Moreover, since the effect of BIBP 3226 is pertussis toxin insensitive, the neuropeptide Y Y_1 receptor is probably not involved because the effect of neuropeptide Y on forskolin-stimulated cyclic AMP accumulation, exerted via the neuropeptide Y Y_1 receptor, in chromaffin cells is pertussis toxin-sensitive (Zhu et al., 1992). However, it should be mentioned that the effect of neuropeptide Y on chromaffin cell inositol phosphate accumulation is not pertussis toxin-sensitive. The effect of neuropeptide Y on inositol phosphate accumulation is most likely unrelated to the effect of BIBP 3226 on secretion since BIBP 3226 blocks the effect of neuropeptide Y on inositol phosphate accumulation (Zheng et al., 1997).

Reports of the action of BIBP 3226 have been primarily concerned with its ability to block the neuropeptide Y Y_1 receptor (Doods et al., 1996). This effect can also be demonstrated in bovine chromaffin cells when a neuropeptide Y-mediated effect, such as inhibition of cyclic AMP accumulation, is being examined (Zheng and Hexum, unpublished). The data presented here demonstrate that BIBP 3226 is also an inhibitor of catecholamine secretion from bovine chromaffin cells, an effect unrelated to its ability to block neuropeptide Y Y_1 receptors in chromaffin cells. This is the first report that BIBP 3226 can act as an agonist, probably at a site on the nicotinic receptor-ion channel complex. This agent should be examined for agonist effects on other systems, particularly when concentrations in the range of 0.1–10 μM are being tested for their ability to antagonize the action of neuropeptide Y.

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