Modulation of Agonist Binding to Human Dopamine Receptor Subtypes 
by L-Prolyl-L-Leucyl-Glycinamide and a Peptidomimetic Analogue

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PLG and PAOPA Modulate Agonist Binding to Dopamine Receptors

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Text pages:  24
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Abstract:       248 words
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ABBREVIATIONS:  DA, 3-hydroxytyramine; EDTA ,ethylenediaminetetraacetic acid; $[^3]$H]-7-
OH-DPAT, (+)-(R)-7-hydroxy-2-(dipropylamino)tetralin; Gpp(NH)p,5'-
guanylylimidodiphosphate; G-protein, guanine nucleotide binding protein; MgCl$_2$, magnesium
chloride, NPA, propylnorapomorphine; PAOPA, 3(R)-[(2(S)-pyrrolidinylcarbonyl)amino]-2-
oxo-1-pyrrolidineacetamide; PLG, L-prolyl-L-leucyl-glycinamide; Tris-HCl,
tris (hydroxymethyl) aminomethane hydrochloric acid.
ABSTRACT

The present study was undertaken to investigate the role of the hypothalamic tripeptide, L-prolyl-L-leucyl-glycinamide (PLG) and its conformationally constrained analogue 3(R)-[(2(S)-pyrrolidinylcarbonyl)amino]-2-oxo-1-pyrrolidineacetamide (PAOPA), in modulating agonist binding to human dopamine (DA) receptor subtypes using human neuroblastoma SH-SY5Y cells stably transfected with respective cDNAs. Both PLG and PAOPA enhanced agonist [3H]N-propylnorapomorphine (NPA) and [3H]quinpirole binding in a dose dependent manner to the DA D_{2L}, D_{2S}, and D_{4} receptors. However, agonist binding to the D_{1} and D_{3} receptors and antagonist binding to the D_{2L} receptors by PLG were not significantly affected. Scatchard analysis of [3H]NPA binding to membranes in the presence of PLG revealed a significant increase in affinity of the agonist binding sites for the D_{2L}, D_{2S}, and D_{4} receptors. Analysis of agonist/antagonist competition curves revealed that PLG and PAOPA increased the population and affinity of the high-affinity form of the D_{2L} receptor, and attenuated 5'-guanylylimidodiphosphate (Gpp(NH)p)-induced inhibition of high-affinity agonist binding sites for the DA D_{2L} receptor. Furthermore, direct NPA binding with D_{2L} cell membranes pre-treated with suramin, a compound that can uncouple receptor/G-protein complexes, and incubated with and without DA, showed that both PLG and PAOPA had only increased agonist binding in membranes pre-treated with both suramin and DA, suggesting that PLG requires the D_{2L} receptor/G-protein complex to increase agonist binding. These results suggest PLG possibly modulates DA D_{2S}, D_{2L}, and D_{4} receptors in an allosteric manner and the coupling of D_{2} receptors to the G-protein is essential in order for this modulation to occur.
INTRODUCTION

The tripeptide L-prolyl-L-leucyl-glycinamide (PLG), also known as melanocyte stimulating hormone release-inhibiting factor, has been demonstrated to possess a variety of pharmacological activities in the central nervous system (Drucker et al., 1994; Reed et al., 1994; Srivastava et al., 1988). A series of earlier clinical studies showed that this tripeptide possessed substantial therapeutic activity in Parkinson’s disease, 3,4-dihydroxy-L-phenylalanine-induced dyskinesia, antipsychotic drug-induced tardive dyskinesia and depression (Barbeau et al., 1978; Ehrensing et al., 1974, 1994). However, PLG’s therapeutic activity still needs much improvement in terms of its efficacy and potency. For example, Barbeau et al. (1978), has previously shown that PLG improved the signs of Parkinson’s disease in some patients, yet, failed to detect significant improvement in other patients. However, these authors did report consistent clinical trends, suggesting that further work on this tripeptide would be beneficial.

Currently, little is known about the mechanistic action of PLG, thus, in view of reports that PLG has demonstrated potential therapeutic effects in many CNS disorders (Mishra et al., 1983, 1986), it would be of interest to study the mechanistic manner in which PLG modulates central DA receptors in order to produce an effective and potent drug for people suffering from such disorders. PLG has also been shown to enhance the binding of various agonists such as apomorphine, 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene (ADTN) and N-propynorapomorphine (NPA) to striatal DA receptors, whereas antagonist binding (haloperidol and spiperone) was unaffected (Bhargava, 1983; Srivastava et al., 1988). Moreover, PLG and its peptidomimetic analogues have been shown to modulate the affinity states of DA receptors possibly by enhancing its interaction with guanine nucleotide binding proteins (G-protein) (Costain et al., 1997; Srivastava et al., 1988).
Previous studies using bovine brain synaptosomal membrane fractions showed that PLG and its peptidomimetic analogues primarily modulate agonist binding to DA D₂ receptors (Srivastava et al., 1988). Brain tissue, however, contains a heterogeneous population of various DA receptor subtypes; therefore, it has not been possible to study the interaction of PLG with individual DA receptors using striatal membranes. Therefore, the present study was undertaken to establish specific DA receptor modulation by PLG and its potent peptidomimetic analogue, PAOPA (see Figure 1), and to further determine whether the coupling of DA receptors to the G-protein is required for such a modulatory effect. To execute this study, human neuroblastoma SH-SY5Y cells were transfected with the cDNA of specific DA receptor subtypes, thus generating stably expressing cell lines. This cell line was used for the following reasons: (i) it is a neuronal cell line that has been shown to display a high rate of DA receptor subtype expression (pmoles/mg protein) upon transfection (Nair et al., 1996; Hillion et al., 2002); (ii) these cells synthesize and release DA, as well as housing tyrosine hydroxylase and other related enzymes; (iii) various G proteins, as well as adenylyl cyclase, phospholipase, GTPase activity, PKA, and PKC are present in this cell line (Kazmi and Mishra 1989; Lambert and Nahorski, 1990). Lastly, although this cell line does not express DA receptors, it does express α₂ adrenergic, opiate (Kazmi and Mishra, 1987; 1989), NMDA (Nair et al., 1996), adenosine A₂ (Salim et al., 2000) and muscarinic receptors (Jope and Song, 1997). The expression of these other receptors in this cell line offers a distinct advantage over other cell lines since it allowed us to compare the effects of PLG on other related G-protein coupled receptors without performing multiple transfections. Overall, in comparison to other cell lines, this cell line is somewhat similar to endogenous brain tissue.
We also investigated whether PLG requires the receptor/G-protein complex in order to induce its modulatory effect through the use of suramin. Suramin behaves like a direct antagonist of heterotrimeric G-proteins (such as Go/Gi) since it competes with the receptor for the docking site on the \( \alpha \)-subunit of the G-protein and can uncouple receptor/G-protein complexes, thus preventing GDP dissociation which is essential for G-\( \alpha \) subunit activation (Chung and Kermode 2004; Beindl et al., 1996; Waldhoer et al., 1998).

Collectively, our results indicate PLG and PAOPA clearly display modulation of agonist binding to the D_{2S}, D_{2L} and D_{4} receptors, whereas, agonist binding to the D_{1} and D_{3} receptors remains unaffected. Additionally, agonist binding to the \( \alpha \)-2- adrenergic receptor (Gi-coupled) remained unaffected by PLG or PAOPA. Moreover, conversion of the higher-affinity agonist binding state of the D_{2L} receptor by 5'-guanylylimidodiphosphate (Gpp(NH)p) was clearly attenuated by PLG and PAOPA. Furthermore, the results suggest that PLG requires the G-protein to be coupled to the D_{2L} receptor to modulate agonist binding.
MATERIALS AND METHODS

Materials

SH-SY5Y cells, (5 passages) were obtained from Dr. A. Blume’s laboratory (Roche Institute of Molecular Biology, NJ). \[^{3}H\] spiperone (50 Ci/mmol), \[^{3}H\] NPA (25 Ci/mmol), \[^{3}H\] quinpirole (40 Ci/mmol), \[^{3}H\] rauwolscine (82 Ci/mmol) and \[^{3}H\] SCH 23390 (52 Ci/mmol) were purchased from Perkin Elmer (Boston). \[^{3}H\] 7-OH-DPAT (160 Ci/mmol) was purchased from Amersham Biosciences (Baie d’Urfe, Quebec). PLG, DA and other routine chemicals, including unlabeled spiperone, (+) butaclamol, and quinpirole were purchased from Sigma Chemical Co. (St. Louis, MO). CHO cells, expressing human DA D3 receptors were also obtained from Sigma. 3(R)-[(2(S)-pyrrolidinylcarbonyl)amino]-2-oxo-1-pyrrolidineacetamide (PAOPA) was synthesized as described previously (Yu et al., 1988). Suramin was obtained from Tocris-Cookson (Ellisville, MO), while ascorbic acid was purchased from BDH Inc. (Toronto, ON).

Cell Culture and Gene Transfection

Human DA D2L receptor cDNA was subcloned into the mammalian expression vector pRC/RSV (Invitrogen, USA) whereas human D4 (cDNA-genomic hybrid, Van Tol et al., 1992), D2S, and D1 receptor cDNAs were cloned into pCD-PS expression vectors (Invitrogen, USA). DNA was prepared using the Flexiprep plasmid isolation kit (Pharmacia, USA). Human neuroblastoma SH-SY5Y cells were grown at 37°C under 5% CO₂ / 95% air in RPMI media supplemented with 10% fetal calf serum, 1 mM glutamine, 50 U ml⁻¹ penicillin and 50 U ml⁻¹ streptomycin. Transfection of cells with respective cDNA was performed with 10 μg of DNA to 3x10^7 cells as previously described by Nair et al (1996). Cells were selected with geneticin
(2 µg/ml) (Life technologies) and single colonies were expanded by limiting dilution to generate a stable transfection system.

**Membrane Preparation and Receptor Binding Assays**

The SH-SY5Y cells, stably expressing cloned human DA receptors, were grown to confluency and membranes were prepared as previously described (Nair et al., 1996). The DA receptor agonist binding assays were carried out as previously described (Nair et al., 1996; Srivastava et al., 1988). The assay buffer contained 50 mM tris (hydroxymethyl) aminomethane hydrochloric acid (Tris-HCl), 5 mM potassium chloride (KCl), 4 mM magnesium chloride (MgCl₂), 1 mM ethylenediaminetetraacetic acid (EDTA) and 120 mM sodium chloride (NaCl) (pH 7.6) with 100 µg of membrane protein in a total volume of 1 ml. Protease inhibitors were added to the assays as a precaution against proteolysis of the membrane protein as well as the tripeptide, PLG and its analogue, PAOPA. The concentration of [³H] NPA used for the D₂S, D₂L, and D₄ receptor assays was 0.25 nM while that for the D₁ receptor was 0.5 nM. For the D₃ receptor binding assay, CHO cell membranes were suspended in the above buffer with the addition of 0.05% ascorbic acid to prevent the degradation of [³H] 7-OH-DPAT (1 nM). Specific binding was defined as the difference between the radioactivity bound in absence and presence of 1 µM (+) butaclamol (D₁, D₂, and D₄ receptors) and 1 µM DA (D₃ receptor). The [³H] quinpirole (2 nM) binding assay conditions were the same as for [³H] NPA except that the assay tubes also contained 120 mM glucosamine. The binding of [³H] rauwolscine to α₂-adrenergic receptors and [³H] SCH23390 to D₁ receptors were carried out as previously described (Srivastava et al., 1988). The contents of the tubes (triplicate) were incubated for 1 hr at 25°C. At the end of the incubation period, assays were terminated by rapid filtration through a Brandel cell harvester (Brandel, Gaithersburg, MD)
and radioactivity bound on filters was counted in a Beckman liquid scintillation counter (model LS5 KTA).

For the $[^3\text{H}]$ spiperone/NPA competition assays, 0.2 nM $[^3\text{H}]$ spiperone and various indicated concentrations of NPA were added to the assay mixture. The competition assays were carried out both in the absence and presence of either 100 $\mu\text{M}$ Gpp(NH)p, 1 $\mu\text{M}$ PLG, or Gpp(NH)p and PLG together.

**Cell Membrane Pre-Treatment with Suramin and DA**

200 $\mu\text{g}$ of cell membrane protein was incubated alone, with 10 $\mu\text{M}$ suramin, and with 10 $\mu\text{M}$ suramin + 100 $\mu\text{M}$ DA for 15 min at 25 °C. The DA was dissolved in 0.1 % ascorbic acid. The membranes were then washed three times to remove any unbound DA and suramin, and the resulting pellet was resuspended and used for the receptor binding assays. The assay buffer contained 50 mM Tris, 5 mM MgCl$_2$, 1 mM EDTA, 0.1 mM DL-Dithiothreitol(DTT), 0.1 mM phenylmethanesulfonyl fluoride (PMSF), 100 $\mu$g/ml bacitracin, and 5 $\mu$g/ml soybean trypsin inhibitor (pH 7.4) with 40 $\mu$g of membrane protein in a total volume of 1 ml, in each assay tube. The concentration of $[^3\text{H}]$ NPA used was 1 nM, whereas the concentration of PLG, and PAOPA used was 10 $\mu$M and 1 nM, respectively. The contents of the tubes (triplicate) were incubated for 1 hr at 25 °C. At the end of the incubation period, assays were terminated by rapid filtration in the same manner described above for other binding assays.
Data Analysis

The data were analysed using GraphPad Prism software (version 4.1) (San Diego, California). The receptor binding data were analysed by weighted non-linear curve fitting for agonist versus antagonist competition curves. The data were analysed for either one or two site populations of binding sites together with statistical analysis comparing the goodness of fit between one or two affinity state models. A two-site model was selected only if a statistically significant improvement of the fit of the data was obtained over a one-site model. The IC50 values (concentration of competing ligands that inhibits the binding of the labelled ligand by 50%) obtained from the competition curves were converted to Ki values (inhibitory constant) using the Cheng and Prusoff equation (Cheng and Prusoff, 1973). The data for Scatchard analysis were analyzed by non-linear regression using GraphPad and t-tests were used to compare differences among values between the experiments. Dose response curves were analyzed by one-way repeated measures of analysis of variance (ANOVA) using GraphPad Prism software, followed by Newman-Keuls post-hoc test. The multiple comparison procedure used is indicated in the appropriate figure legend.
RESULTS

PLG and the peptidomimetic analogue PAOPA increase [³H] NPA binding to the DA D₂L, D₂S, and D₄ receptor subtypes.

To determine whether PLG and PAOPA increase agonist binding to specific DA receptor subtypes, receptor binding assays were carried out with the agonist, [³H] NPA, for various DA receptor subtypes. The results outlined in Figure 2, clearly demonstrate that both PLG and PAOPA increase agonist binding to the D₂L, D₂S, and D₄ receptors, whereas agonist binding to the D₁ ([³H] NPA) and D₃ ([³H] 7-OH-DPAT) receptors were not statistically significantly affected. The stimulatory effect is concentration dependent and displays a bell shaped curve. The bell shaped curve is consistent with the previously reported findings for in vivo animal experiments (Haran and Kastin, 1986; Costain et al., 1999; Mishra et al., 1997) and human clinical studies utilizing PLG (Drucker et al., 1994; Ehrensing et al., 1994). In comparison to PLG, PAOPA displayed similar efficacy and ranged from being 10 to 1000 times more potent.

PLG and PAOPA increase [³H] quinpirole binding to the DA D₂L, D₂S, and D₄ receptors.

To determine whether PLG or its potent analogues also increase the binding of another D₂ receptor family agonist, the binding of [³H] quinpirole was also examined in these cultures. As shown in Figure 3a and 3b, both PLG and PAOPA significantly increased the binding of [³H] quinpirole to the D₂L, D₂S, and D₄ receptors. However, although PAOPA displayed a similar efficacy to PLG, it was found to be 1000 times more potent for the D₂L receptor, 100 times more potent for the D₂S receptor and displayed the same potency for the D₄ receptor.
PLG decreases the equilibrium dissociation constant (K_d) for [^3H] NPA binding to the D_{2L}, D_{2S} and D_{4} receptors.

In order to determine whether the increase in agonist binding was due to an increase in B_{max} or a decrease in K_d, Scatchard analysis of [^3H] NPA binding in the absence and presence of 1 µM PLG was performed on membranes collected from cultures transfected with the D_{2L}, D_{2S}, and D_{4} receptor. The data shown in Figure 4 reveals a single class of binding sites with a K_d of 0.20±0.015 nM (mean ± standard error of the mean (SEM) and a total number of binding sites of 1259±104 fmol/mg protein. When D_{2L} transfected membranes were treated with PLG (1 µM) Scatchard plot analysis illustrated a significant decrease (P<0.01) in the K_d value (0.09±0.004 nM) without any significant increase in B_{max} (1473±109 fmol/mg protein). Similarly, both PLG and PAOPA affected the equilibrium dissociation constant of [^3H] NPA for the D_{2S} and D_{4} subtypes (see Figure 5 and 6). The Scatchard plot analysis shown in Figure 5 and 6 revealed a K_d value of 0.25±0.020 nM and a B_{max} of 470.0±30.0 fmol/mg protein in the absence of PLG for the D_{2S} receptor. When the membranes were treated with PLG, there was a significant decrease (P<0.01) in the K_d value (0.10±0.009 nM), without significantly affecting the B_{max} (488.0±40.0 fmol/mg protein). Similarly, the K_d value obtained for the D_{4} receptor subtype in the absence of PLG was 0.35±0.026 nM with a significant decrease (P<0.05) in the presence of PLG (0.14±0.013 nM). The B_{max} values, however, did not show a significant difference in the absence and presence of PLG, (1680±170 fmol/mg protein with out PLG and 1710±195 fmol/mg protein with PLG).
PLG does not affect antagonist [3H] spiperone binding to the DA D2L receptors in transfected cells.

To determine whether antagonist binding is affected by PLG in D2 transfected cells, binding assays with [3H] spiperone were carried out. In contrast to agonist, [3H] NPA, binding to the D2L, D2S, and D4 receptors, the binding of [3H] spiperone was not affected by the addition of PLG. Representative binding parameters obtained for the D2L receptors demonstrated that the $K_D$ and $B_{max}$ values (Scatchard analysis) remained unchanged for both control ($K_D$ 0.09±0.005 nM; $B_{max}$ 1214±122 fmol/mg protein) and PLG-treated membranes ($K_D$ 0.07±0.003 nM; $B_{max}$ 1256±128 fmol/mg protein). These results are consistent with other reports in which no changes in [3H] spiperone binding in striatal membranes were observed (Bhargava, 1983; Chiu et al., 1981a; Mishra et al., 1990; Srivastava et al., 1988). Similar observations were made for the D2S and D4 receptors (data not shown) thereby extending these results to other D2 receptor family members.

PLG and PAOPA attenuate Gpp(NH)p-induced conversion of high-affinity agonist binding state to low-affinity state in D2L receptors.

In order to establish whether the high-affinity agonist state of the D2 receptor is affected by PLG, [3H] spiperone/NPA competition experiments were carried out in the presence of PLG and Gpp(NH)p. The representative results from the [3H] spiperone/NPA competition for the D2L receptor are shown in Figure 7. Analysis of the competition curves revealed that NPA discriminates between two D2L receptor affinity states which are labelled by [3H] spiperone. The two states, agonist high-affinity and agonist low-affinity, were present in approximately equal proportions, 53% and 47%, respectively, in the control transfected cell membranes, however,
significantly differed with respect to their $K_i$ values (0.065 nM for the high-affinity state and 94 nM for the low-affinity state).

In the PLG-treated $D_{2L}$ receptor membranes, there was a leftward shift in the competition curve with an almost 3-fold decrease in $K_i$ for the high-affinity agonist binding site without any significant change in the $K_i$ for the low-affinity site. The ratio of high- to low-affinity sites in the PLG-treated membranes significantly increased from 1.12 (control) to 2.70 (Table 1).

The non-hydrolyzable analogue of GTP, Gpp(NH)p, caused a significant rightward shift in the competition curve for the $D_{2L}$ receptor; this decreased the proportion of high-affinity binding sites to 18% with a $K_i$ of 0.15 nM. However, the conversion of the high- to low-affinity state was significantly attenuated by PLG. For example, the percentage of receptors in the high-affinity state was 34% with a $K_i$ of 0.040 nM in the PLG-treated membranes (Table 1). Yet, the affinity of the receptors remaining in the low-affinity state was unaffected (Table 1). PAOPA demonstrated similar results to PLG (Table 2), yet, PAOPA was almost 1000-times more potent than PLG.

**PLG does not affect the conversion of the high-affinity state to the low-affinity state by Gpp(NH)p for the DA $D_1$ and $\alpha_2$-adrenergic receptors.**

To establish the specificity of PLG with respect to modulating the $D_2$ receptor subtype, DA $D_1$ and $\alpha_2$-adrenergic receptors were also utilized. The $D_1$ receptor antagonist/agonist competition curve using $[^3H]$ SCH23390 (antagonist) and SK&F38393 (agonist) revealed a high- and low-affinity state of agonist binding, although the binding parameters were unaffected by the addition of PLG. The values obtained from controls were: $K_{H}$, 3.00±0.25 nM; $K_L$, 96.0±7.0 nM; percent $R_H$, 30.0±2.5; percent $R_L$, 70.0±9.0. Similar values were obtained from the PLG-treated group.
and were not significantly different (K_H, 2.60 ± 0.18 nM; K_L, 92.0 ± 8.0 nM; percent R_H, 34.0 ± 5.0; percent R_L, 66.0 ± 9.0).

The α_2-adrenergic receptor antagonist/agonist competition curves, using [³H] rauwolscine as an antagonist and BHT-920 as the agonist, displayed a high-affinity and low-affinity state of agonist binding as previously reported in this cell line (Kazmi and Mishra, 1989). The binding parameters were also unaffected by PLG or even by its highly constrained analogue, PAOPA. The values obtained in the control membranes for PLG (K_H, 2.60±1.1 nM; K_L, 102±13 nM; percent R_H, 43.0±3.2; percent R_L, 57.0±4.5) were not significantly different from those obtained in the PLG-treated membranes (K_H, 2.48±0.051 nM; K_L, 102±13.0 nM; percent R_H, 45.0±4.0; percent R_L, 55.0±5.1). Even the highly potent, conformationally constrained analogue, PAOPA, did not alter the affinity state of the α_2-adrenergic receptors, with the control values being K_H, 2.9±0.21 nM; K_L, 107±14 nM; percent R_H, 43.0±3.0; percent R_L, 57.0±5.0 and the PAOPA treated values being K_H, 2.7±0.30 nM; K_L, 110±11 nM; percent R_H, 46.0±4.0; percent R_L, 54.0±4.7.

**Effect of Suramin on PLG induced increase in agonist binding**

To establish whether PLG and PAOPA display modulatory effects on agonist binding to D_2 receptors in the absence of G-protein coupling, we investigated agonist binding in the absence and presence of suramin. Suramin is a polysulfonated naphthylurea and is considered to uncouple the receptor from the G-protein that results in decreased high-affinity agonist binding (Chung and Kermode, 2004; Nakata, 2003; Nickolls and Strange, 2003). Both PLG and PAOPA, as shown in Figure 8, were unable to modulate [³H] NPA binding to D_2L receptors in the presence of suramin. PLG and PAOPA clearly increased agonist binding in membranes pre-
treated with suramin and DA, but had no effect on the membranes treated with suramin alone. Furthermore, to establish whether this lack of PLG modulation is due to a direct competition with suramin, a competition experiment was performed. As indicated in Figure 9, the competition curve revealed that PLG does not compete with suramin for the receptor binding site.
DISCUSSION

The main findings of the present paper are first that both PLG and a conformationally constrained analogue (see Figure 1) increase agonist binding to specific DA receptor (D_{2S}, D_{2L}, and D_{4}) subtypes in the D_{2} receptor family (see Figure 2 and 3). Second, this increase in agonist binding is dependent upon D_{2} receptor/G-protein coupling (see Figure 8).

The results of this study clearly provide evidence that PLG and PAOPA enhance agonist binding (NPA and quinpirole) to the DA D_{2L}, D_{2S} and D_{4} receptors, in a manner that is selective for the agonist high-affinity state of the receptor. The ineffectiveness of PLG and PAOPA on spiperone (D_{2} receptor antagonist), SCH 23390 (D_{1} receptor antagonist) and the α_{2}-adrenergic receptor confirms the specificity of both these peptides toward D_{2} receptor agonist binding. Furthermore, results from the D_{1}, D_{3} and α_{2}-adrenergic receptor binding ascertain that both agonist and antagonist binding to these receptors is unaffected by PLG and PAOPA.

The results from [^{3}H] NPA and [^{3}H] quinpirole binding to the DA D_{2L}, D_{2S}, and D_{4} receptors clearly reveals that the effect of PLG and PAOPA are dose-dependent with a maximal effect at 0.1 to 1.0 µM for PLG, and 0.001 to 0.1 µM for PAOPA. This dose response corroborates the phenomenon of a “therapeutic window” in which the pharmacological response for PLG can be elicited in animal models (Costain et al., 1999; Drucker et al., 1994; Mishra et al., 1997; Sharma et al., 2003) and human clinical trials (reviewed by Mishra et al., 1986).

The PLG- and PAOPA-induced increase in agonist binding for the D_{2} receptor is selective for the high-affinity state; this is evident from the antagonist/agonist competition experiments carried out for the D_{2L}, D_{2S} and D_{4} receptors (Table 1), where a significant increase in the affinity and population of high-affinity binding sites was observed for [^{3}H] NPA. The number of low-affinity sites for [^{3}H] NPA decreased as a result of PLG and PAOPA treatment.
without a significant change in affinity. Thus, these results suggest a conversion from the D₂ receptor low-affinity form to the high-affinity form for agonists in the presence of PLG and PAOPA.

The increase in the affinity of the high-affinity agonist binding site is consistent with the Scatchard analysis of [³H] NPA binding for the D₂L, D₂S, and D₄ receptors in PLG-treated membranes since the [³H] NPA concentrations used (up to 1 nM) have been reported to selectively label the high-affinity form of the DA D₂ receptor (Titeler and Seeman, 1979). High and low-affinity states of the D₂ receptors are an indication of coupling and non-coupling, respectively, of the receptor with the G-protein, Gᵢₐβγ GDP / Goᵢβγ GDP. Therefore, the observed increase in the proportion and affinity of the binding sites following PLG and PAOPA treatment may be due to an increase in the association of the D₂ receptor with Gᵢₐβγ GDP.

The enhancement of the affinity and proportion of high-affinity agonist binding sites by PLG and PAOPA, and their attenuation on the effect of Gpp(NH)p (Figure 1, Table 1 and 2), demonstrates an increased interaction between DA D₂ receptors and G-proteins. PLG was unable to increase agonist binding in the presence of suramin which uncouples the receptor/G-protein complex; this suggests that PLG will only interact with D₂ receptors when they are coupled to the G-protein α–βγ subunits. However, the antagonistic effect of suramin on G-proteins can be reversed by the addition of agonist (Beindl et al., 1996). In this case, the increased concentration of DA (100 µM) reversed the inhibitory effect of suramin because the agonist-liganded receptor competes with suramin for binding to the G-protein. Increasing the number of active receptors in the membrane by increasing agonist occupancy can overcome the inhibitory effect of suramin on the receptor/G-protein complex (Beindl et al., 1996). Additionally, the suggestion that PLG requires the receptor/G-protein complex may be part of
the reason for the lack of significant PLG modulation associated with the D3 receptor subtype. In contrast to the D2 and D4 receptor subtypes, the receptor/G-protein interaction associated with the D3 receptor subtype is weaker since GTP does not modulate high affinity agonist binding to the D3 receptor (Filteau et al., 1999; McAllister et al., 1993). It is also possible that PLG cannot modulate the D3 receptor for reasons similar to that of GTP; further research is required to explore this possibility.

Previous studies have utilized bovine striatal membranes, which contains a heterogeneous population of DA receptors, as a means of testing the action of PLG on the DA receptor. However, since the mechanism of the interaction between PLG and the DA receptor is still not clear, we have attempted to study this interaction in cultures stably transfected with individual DA receptor subtypes, which provided a model based analysis/interpretation of PLG’s modulatory effect. This model facilitated specific attention to binding parameters as well as experimental manipulation to identify the role of high vs. low affinity and G-protein coupling.

Additionally, the importance of the D2L receptor/G-protein complex is indicative of the possible site of interaction with PLG by which this endogenous neuropeptide may be modulating and increasing the number and affinity of the D2L receptors in the high affinity state. We suggest a mechanism which proposes that PLG causes a conformational change in the receptor, presumably involving a putative PLG binding site on the D2 receptor (Chiu et al., 1983a, b); this would lead to an increased association of the receptor with the G-protein (G_i) by adjusting the equilibrium between the receptor, G-protein and receptor/G-protein complex (Costain et al., 1997). Such a mechanism would explain the enhancement of high-affinity agonist binding and the effect of PLG opposite to that of Gpp(NH)p.
If PLG is acting at a distinct site on the D₂ receptor, it is possible that PLG is acting as an allosteric ligand. The conformational change is frequently synonymous with the term allosteric modulation because allosteric modulators are described as modulators that occupy sites other than the “primary” site of ligand binding, the orthosteric site. Both DA D₂ and D₄ receptors have been shown to be allosterically modulated by amilorides and zinc (reviewed by May and Christopoulos, 2002). Furthermore, many receptors of the G protein coupled receptor superfamily are allosterically modulated. Allosteric modulators modify receptor conformation to cause a change in the binding of the orthosteric ligand (May and Christopoulos 2003; Kenakin, 2004); this is what PLG does precisely; it increases the binding of an agonist (orthosteric ligand) to D₂ receptors, perhaps in an allosteric manner. However, unlike zinc or amilorides, PLG requires the D₂ receptor to be coupled to the G-protein and therefore, the molecular mechanisms of allosteric modulation for PLG are more distinct than other modulators. The simplest mechanism that describes an allosteric interaction between two ligands binding at distinct sites on a receptor is referred to as the ternary complex model (TCM) (Kenakin, 2004). In this model (Figure 10), each ligand binds to the receptor with its own affinity constant with the symbol, α, acting as the cooperative factor that is used to quantify the magnitude of the change in affinity of one ligand that is caused by the binding of the second ligand. The ability of the allosteric modulator (PLG) to cause a change in affinity for the agonist (NPA or quinpirole) binding at the orthosteric site relates to the degree in which the modulator (PLG) induces a change in the receptor conformation. The TCM model has already successfully quantified the behaviour of several other allosteric modulators of G-protein coupled receptors (Christopoulos, 2002).

The findings reported in this study show important implications with regards to the interaction of PLG and conformational pharmacology of DA D₂ receptors. First, the presence of
an endogenous neuropeptide site of allostERIC modulation should allow for the synthesis of higher affinity modulators, such as PAOPA shown here, with increased selectivity. Second, PLG analogues such as PAOPA provide additional tools for probing the conformational pharmacology of plasma membrane bound G-protein coupled DA D2 receptors. Third, PLG displays no effect in the absence of orthosteric ligands, therefore the normal spatial and temporal pattern of physiological signal transduction and termination is maintained, with the only effect of PLG is being able to increase the pattern of signaling as suggested by Christopoulos and Kenakin (2002). Finally, there is very little possibility of toxic effects of modulators such as PLG or PAOPA since there is a ceiling to their effects after a maximal dose. Interestingly, this modulatory effect is reduced at higher concentrations.

In conclusion, we have unequivocally shown the selective modulation of the human D2S, D2L, and D4 DA receptor subtypes by PLG and provided a theoretical mechanistic action of PLG; this can now be used to perform even more detailed studies on this tripeptide which may lead to the development of a more effective and potent drug for the treatment of neurological disorders such as Parkinson’s disease and antipsychotic drug-induced tardive dyskinesia.
ACKNOWLEDGEMENTS

We are grateful to Ms. Cia Barlas for excellent suggestions and discussions.
REFERENCES


Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 percent inhibition (I50) of an enzymatic reaction. Biochem Pharmacol 22:3099-3108.


FOOTNOTES

This work was supported by National Institute of Health (USA) grant NS20036.

* In memory of Dr Hyman B. Niznik
**LEGENDS FOR FIGURES**

**Figure 1** Structure of hypothalamic tripeptide L-prolyl-L-leucyl-glycinamide (PLG) and its conformationally constrained analogue 3(R)-[(2(S)-pyrrolidinylcarbonyl)amino]-2-oxo-1-pyrrolidineacetamide (PAOPA).

**Figure 2** Stimulation of agonist binding to DA receptors in transfected cell membranes by PLG (a) and PAOPA (b). The ligands used were [3H] NPA for D1, D2 and D4 receptors and [3H] 7-OH-DPAT for D3 receptors. Respectively transfected human neuroblastoma SH-SY5Y cell membranes were used for D1, D2 and D4 receptors, whereas D3 receptor binding was carried out in CHO cell membranes. The data were analyzed by one way analysis of variance (ANOVA) followed by the Newman-Keuls post-hoc test. Values are given as percent increase in binding observed in the absence of PLG and PAOPA. Results are expressed as mean ± S.E.M. of four independent experiments. *P< 0.01, specifically in figure 2a log concentrations: -8 to -6 for D2 and -7 for D4 P<0.01. In figure 2b log concentrations: -10 to -8 for D2 and -8 to -7 for D4 P<0.001.

**Figure 3** Stimulation of [3H] quinpirole binding to D2L, D2S and D4 receptors by PLG (1 µM) (a) and PAOPA (1 nM) (b). The data were analyzed by one way analysis of variance (ANOVA) followed by Newman-Keuls post-hoc test. Each value is an average of four to five separate experiments ± S.E.M. *P<0.05 specifically, in figure 3a points at log concentrations: -9 to -5 for D2L, -8 to -6 for D2S and -9 to -6 for D4, P< 0.001; -9 for D2S and -5 for D4 had P<0.01. In figure 3b, log concentrations: -11 to -7 for D2L, -9 to -7 for D2S and -9 to -7 D4, P< 0.001; -6 for D2L P< 0.01.
Figure 4 Saturation and Scatchard plot of $[^3\text{H}]$ NPA binding to control and PLG-treated membranes for the D$_{2L}$ receptor subtype. The Scatchard analysis was performed with concentrations of $[^3\text{H}]$ NPA ranging from 0.01 nM to 2 nM. Data were analysed by non-linear regression analysis for either a one or two site population of binding sites with statistical analysis comparing the goodness of fit between one or two sites. The data yielded a single class of binding sites. Results are an average of triplicate determinations and are representative of three such experiments with similar results.

Figure 5 Saturation and Scatchard plot of $[^3\text{H}]$ NPA binding to control and PLG-treated membranes for the D$_{2S}$ receptor subtype as performed for Figure 4. Results are an average of triplicate determinations and are representative of three such experiments with similar results.

Figure 6 Saturation and Scatchard plot of $[^3\text{H}]$ NPA binding to control and PLG-treated membranes for the D$_4$ receptor subtype as performed for Figure 4. Results are an average of triplicate determinations and are representative of three such experiments with similar results.

Figure 7 Competition curves of $[^3\text{H}]$ spiperone (0.2 nM) versus NPA in control and PLG-treated membranes with and without Gpp(NH)p. Analysis of the curves using GraphPad Prism (version 2.10) revealed two binding sites. The binding parameters are provided in Table 1. Results are expressed as mean ± S.E.M. of three separate experiments. The concentrations used for PLG and Gpp(NH)p were 1 µM and 100 µM, respectively.
**Figure 8** Stimulation of $[^3]H$ NPA binding to the $D_{2L}$ receptor in the absence and presence of PLG (10 $\mu$M) or PAOPA, (1 nM) with respect to untreated membranes (control). Results are an average of triplicate determinations (*P<.05) and are expressed as an average of three separate experiments ± S.E.M. Stimulation of $[^3]H$ NPA binding (PLG or PAOPA treatment) to the $D_{2L}$ receptor in suramin treated membranes in the absence and presence of DA. Results are an average of triplicate determinations (*P<0.001) and are expressed as an average of three separate experiments ± S.E.M. Statistical comparisons were made between membranes treated with DA and their respective groups treated in the absence of DA.

**Figure 9** Competition curves of $[^3]H$ NPA with suramin in the absence and presence of PLG. Each value is an average of three to four separate experiments ± S.E.M. The Hill Plot was used to fit the graphical data.

**Figure 10** Ternary complex model for G-protein coupled receptors as referenced by Christopoulos (2002), where the allosteric modulator, PLG, binds to a site that is topographically distinct from the orthosteric site that is used by the agonist, N (NPA); (D$_{2L}$=D$_{2L}$ Receptor).
Table 1. Modulation of $[^3H]$ Spiroperidol/NPA Competition at D$_{2L}$ Receptors by PLG

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<tr>
<td></td>
<td>$K_H$ (nM)</td>
<td>$K_L$ (nM)</td>
<td>Percent $R_H$</td>
<td>Percent $R_L$</td>
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<td>-Gpp(NH)$_p$</td>
<td>0.065 ± 0.003</td>
<td>94.0 ± 5.0</td>
<td>53.0 ± 4.0</td>
<td>47.0 ± 5.0</td>
<td>1.12 ± 0.13</td>
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<tr>
<td>+Gpp(NH)$_p$</td>
<td>0.15 ± 0.01</td>
<td>88.0 ± 4.0</td>
<td>18.0 ± 2.0</td>
<td>82.0 ± 6.0</td>
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<tr>
<td>-Gpp(NH)$_p$</td>
<td>0.020 ± 0.001 (a)</td>
<td>78.0 ± 5.0</td>
<td>73.0 ± 6.0 (a)</td>
<td>27.0 ± 3.0</td>
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<tr>
<td>+Gpp(NH)$_p$</td>
<td>0.040 ± 0.002 (b)</td>
<td>92.0 ± 8.0</td>
<td>34.0 ± 2.5 (b)</td>
<td>66.0 ± 11</td>
<td>0.55 ± 0.040 (b)</td>
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Competition data as shown in Figure V, were analyzed using GraphPad Prism software. $K_H$ and $K_L$ represent the inhibition constant ($K_i$) of agonist, calculated from the high-affinity and low-affinity component of $[^3H]$ spiroperidol binding respectively. Percent $R_H$ and Percent $R_L$ are respectively, the percentage of sites in high- or low-affinity form for the agonist. $R_{H/L}$ is the ratio of the two percentages. Each value is an average of three to four separate experiments ± S.E.M., carried out in duplicate or triplicate. Differences from the respective control groups are indicated as follows: (a) $P<0.01$; (b) $P<0.05$. The concentration of PLG used was 1 µM.
Table 2. Modulation of [$^3$H] Spiroperidol/NPA Competition at D$_{2L}$ Receptors by PAOPA

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<tr>
<td>-Gpp(NH)p</td>
<td>0.071 ± 0.004</td>
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<tr>
<td>+Gpp(NH)p</td>
<td>0.18 ± 0.01</td>
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<tr>
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<tr>
<td>-Gpp(NH)p</td>
<td>0.030 ± 0.002 (a)</td>
</tr>
<tr>
<td>+Gpp(NH)p</td>
<td>0.050 ± 0.003 (b)</td>
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Competition data similar to that shown in Figure V, were analyzed using GraphPad Prism software. $K_H$ and $K_L$ represent the inhibition constant ($K_i$) of agonist, calculated from the high-affinity and low-affinity component of [3H] spiroperidol binding respectively. Percent $R_H$ and Percent $R_L$ are respectively, the percentage of sites in high- or low-affinity form for the agonist. $R_H/R_L$ is the ratio of the two percentages. Each value is an average of three to four separate experiments ± S.E.M., carried out in duplicate or triplicate. Differences from the respective control groups are indicated as follows: (a) $P<0.01$; (b) $P<0.05$. The concentration of PAOPA used was 1 nM.
Figure 1
Figure 2a
Figure 2b
Figure 3a
Figure 3b
Figure 4
Figure 5
Figure 6
Figure 7
Figure 9
Figure 10