0026-895X/88/010078-06$02.00/0

MOLECULAR PHARMACOLOGY, 33:78-83

Correlation of α- and β-Rotameric Forms of 2-Substituted Octahydrobenzo[*f*]quinoline Dopamine Congeners with High and Low Affinity States of the Anterior Pituitary Dopamine Receptor and Prolactin Inhibition

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Received August 14, 1987; Accepted October 26, 1987

SUMMARY

The flexible dopamine (DA) molecule exists in one or the other of its two conformational extremes (α- or β-rotamer) and its receptor in the anterior pituitary gland exists in a high and a low affinity state. A series of novel, rigid DA congeners (2-substituted octahydrobenzo[*f*]quinolines) was synthesized and used to investigate the conformation of DA preferred by its anterior pituitary receptor and the significance of recognition of the two affinity states to the inhibition of prolactin (PRL) secretion. Analysis of competition curves of congeners for [^{3}H]spiperone binding to bovine anterior pituitary membranes was used to calculate affinity constants. Congeners in the β-rotamer conformation showed a biphasic competition curve as observed for DA. The curves were resolved into high (nM) and low (μM) affinity binding sites. This biphasic binding could be converted to monophasic low affinity binding in the presence of a nonhydrolyzable GTP analog. The congeners in the α-rotameric conformation showed monophasic low affinity binding. The potency of congeners to suppress PRL release was evaluated in cell cultures of dispersed bovine anterior pituitary. Congeners recognizing the high affinity binding site were 100-fold more potent in suppressing PRL release than those recognizing only low affinity binding sites. Dihydroxy congeners versus monohydroxy congeners and cyanomethyl group substituted versus methylthiomethyl substituted congeners occupied greater proportions of high affinity binding sites. Increasing proportions of high affinity sites occupied increased the potency of the congener to suppress PRL release. These results suggest that the β-rotameric conformational extreme of DA is preferred by its receptor in the anterior pituitary gland and that the high affinity state of this receptor is functionally important in mediating the inhibition of PRL secretion.

In the AP gland, agonist occupancy of the DA receptor initiates a series of intracellular events which lead to the inhibition of PRL secretion. Radioligand binding studies have revealed the existence of a single homogeneous class of AP DA receptors (1, 2) which, based upon biochemical and pharmacological criteria, belong to the D-2 category of DA receptors (3, 4). In membrane preparations of the AP, this DA receptor exists in either a high (nM) or a low (μM) affinity state for its ligand. The two affinity states are interconverted by guanine nucleotides and are not distinguished by most DA antagonists (5, 6). Furthermore, the flexible DA molecule exists in two conformational extremes (α- or β-rotamer) and is believed to interact effectively with its receptor in one or the other of these conformational extremes (7).

It is still unclear as to the relationship between the two conformational extremes of the DA molecule and the two affinity states of the DA receptor and the role(s) each may play in mediating the inhibition of PRL secretion. Since the concentration of DA in hypophysial portal blood reaching the AP is in the nM range (8, 9), it would appear that the high affinity state of the receptor plays a key role in the physiological regulation of PRL secretion. Furthermore, a large number of synthetic DA agonists which demonstrate high affinity binding inhibit PRL secretion with potencies in the nM range (10). The inhibition of adenylate cyclase, implicated in the control of PRL release (11–14), is also highly correlated with the high affinity binding state (15).

In this study we have synthesized a series of novel, rigid DA congeners (2-substituted octahydrobenzo[*f*]quinolines), investigated the ability of these congeners to compete for binding of the DA antagonist SPIR to membrane preparations of the

This work was supported by National Institutes of Health Grants GM31661 and AM 07256.

[1] J. C. Craig, S. M. Torkelson, P. R. Findell, and R. I. Weiner, submitted for publication.

ABBREVIATIONS: AP, anterior pituitary; DA, dopamine; PRL, prolactin; SPIR, spipoperidol; Hepes, 4-(2-hydroxyethyl)-2-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Gpp(NH)p, guanosine 5′-(β,γ-inido)triphosphate.

bovine AP gland, and compared this to their ability to inhibit PRL release in primary AP cell cultures of the same species.

The significance of this series of congeners is that the pharmacophore of DA is held rigid in either the α- or β-rotameric conformation. Dependent upon the conformation, these congeners recognize different proportions of the high and low affinity states or the DA receptor in bovine AP membranes. These novel congeners are therefore useful to investigate the conformation of DA preferred by its AP receptor and to determine the significance of the recognition of the different affinity states of the DA receptor to the inhibition of PRL secretion.

Materials and Methods

Structures of the 2-substituted octahydrobenzo[*f*]quinoline DA congeners. The structures of the eight 2-substituted octahydrobenzo[*f*]quinolines prepared in this study are shown in Fig. 1. Four are the β-rotameric forms (i.e., hydroxyls in the 9- or 8,9-position; designated B.1–B.4) and four are α-rotameric DA congeners (i.e., hydroxyl groups in either the 7- or 7,8-position of the molecule; designated A.1–A.4). Both α- and β-rotameric forms were synthesized with either: (i) a methylthiomethyl group (—CH_2SCH_3; designated with odd numbers) at the 2-position; or (ii) a cyanomethyl group (—CH_2CN; designated with even numbers) as the 2-position substituent. The backbone skeletons of each molecule are identical. The methods utilized to synthesize these DA congeners have been reported previously.[1] It must be pointed out that each congener is a racemate and only one enantiomer for each congener is drawn in Fig. 1.

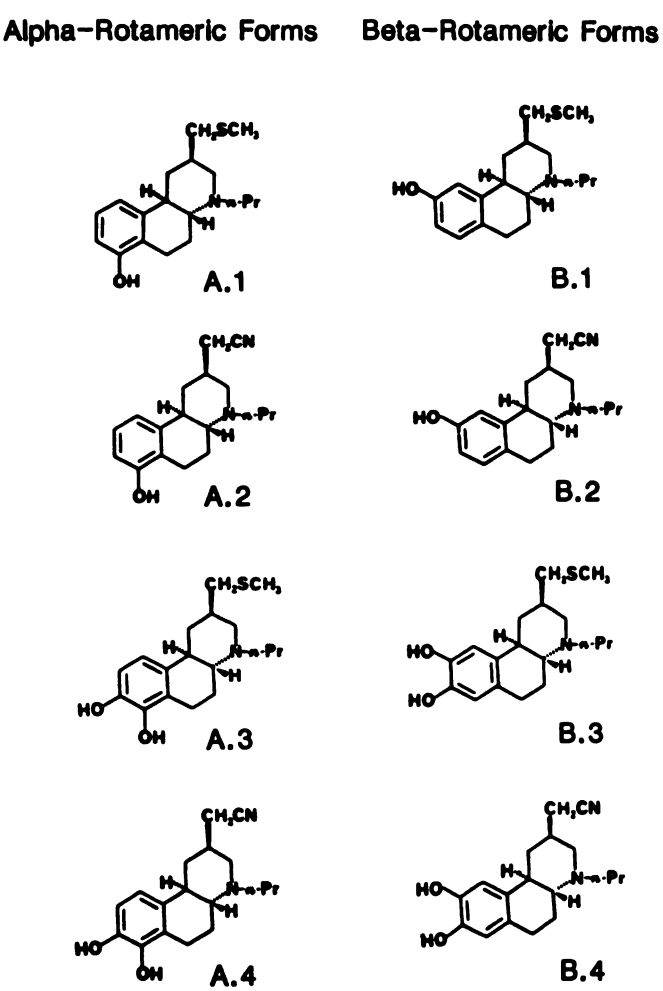

Fig. 1. Structures of the 2-substituted octahydrobenzo[*f*]quinolines prepared and utilized in this study. The congeners are divided into α and β-rotameric conformations and only the *trans*-enantiomers are drawn.

Radioligand binding studies. Freshly removed steer pituitary glands were collected at a local slaughterhouse (McDermott Meat Packing, Berkeley, CA), placed in ice-cold Hanks' calcium, magnesium-free media with 25 mM Hepes buffer (pH 7.2), and transported to the laboratory. The pituitary was then dissected free of dura mater and the anterior and posterior lobes were separated. The anterior lobes were homogenized in 2 volumes (w/v) of ice-cold 0.32 M sucrose containing 15 mM Tris buffer and 1 mM EDTA (pH 7.4), first with a Polytron homogenizer (Brinkmann Instruments, Westburg, NY; three 10-sec intervals at high speed) and then with a Teflon homogenizer (10 strokes at 500 rpm). The homogenate was centrifuged at 1000 × *g* for 15 min and the pellet was discarded. Aliquots of the crude homogenate were stored frozen at −60° for use within 1 month.

At the start of each binding assay, an aliquot of crude homogenate was thawed and then centrifuged at 13,000 × *g* for 15 min. The resulting pellet was discarded and the supernatant centrifuged at 145,000 × *g* for 45 min. All centrifugations were performed at 4°. The particulate pellet was resuspended in the buffer used for binding assays with a glass-glass homogenizer (10 strokes by hand). The assay buffer employed contained 50 mM Tris, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Kcl, 120 mM NaCl, 0.1% ascorbic acid, and 15 μM nialamide.

The particulate preparation was incubated at 37° for 30 min to inactivate monoamine oxidase. Then, 0.1–0.2 mg of membranes was returned to room temperature (22°) and incubated for 60 min with [^{3}H]SPIR (New England Nuclear, Boston, MA; SA 26.4 Ci/mmol), a potent DA antagonist. Preliminary saturation curves with [^{3}H]SPIR (0.04–30 nM, 12 concentrations) were first conducted to determine the dissociation constant of the radioligand. Competition binding studies were done with 1.0–1.5 nM [^{3}H]SPIR in the presence of increasing concentrations of the indicated DA congener (using 14–19 concentrations ranging from 100 μM to 0.1 nM). Nonspecifically bound [^{3}H]SPIR was determined by performing parallel incubations in the presence of a 1000-fold excess of *d*-butaclamol (Ayerst), a potent, stereoselective DA antagonist. Competition binding assays were also carried out in the presence of a 100 μM concentration of the nonhydrolyzable GTP analog, Gpp(NH)p (Sigma Chemical Co., St. Louis, MO).

The incubations were terminated by adding 4 ml of ice-cold buffer to each tube and immediately filtering the contents under vacuum on Whatman GF/c glass filter fibers (Whatman, Inc., Clifton, NJ). The tubes were rinsed twice with 4 ml of buffer and the filters were washed with an additional 8 ml of buffer. The particulate-bound [^{3}H]SPIR trapped on the filters was quantitated by drying the filters and counting them in Hydrofluor (National Diagnostics, Manville, NJ) in a Packard (Downers Grove, IL) Tri-Carb liquid scintillation counter with a machine efficiency of 35%

Competition curves were analyzed by an iterative, nonlinear least squares curve-fitting procedure according to a one- or two-affinity state model for ligand-receptor systems. This computer-assisted analysis provides estimates for the affinity of the congener for the different states of the receptor ($K_{i\,high}$ and $K_{i\,low}$) and the proportions of these states. The inhibition constant (K_i) was determined by the relationship: $K_i = IC_{50}/[1 + (L/K_D)]$ (17), where IC_{50} = competitor concentration that inhibited [^{3}H]SPIR binding by 50%, L = concentration of [^{3}H]SPIR used in the assay (1.0–1.5 nM), and K_D = the dissociation constant of [^{3}H]SPIR for its binding sites (0.16 nM). Statistical analysis comparing "goodness of fit" between the one- and two-affinity state models was also performed and used to determine the appropriate model for the congener being examined (18).

Assay of PRL-inhibiting activity of congeners in bovine AP cell primary cultures. Bovine AP tissue, collected and prepared as previously described, were minced into pieces 2–4 mm in diameter and washed repeatedly in Hanks' calcium, magnesium-free media containing 25 mM Hepes, penicillin-streptomycin (20 units/ml), and fungizone (2.5 μg/ml).

Media products for tissue culture were obtained from Cell Culture

Facility, University of California, San Francisco. The minced tissue was then incubated at 37° for 40 min in the above media containing 0.1% collagenase [Worthington Enzymes (Malvern, PA) type I], 0.1% hyaluronidase, and DNase I (4 μg/ml, Sigma). The suspension was gently stirred in a 50-ml spinner flask on a magnetic stirrer at slow speed. The tissue minces were then mechanically dispersed by trituration using a 10-ml serological pipette. The cell suspension was filtered through cheesecloth and the filtrate was centrifuged at 150 × *g* for 10 min. The cell pellet was then resuspended in Dulbecco's modified Eagle's medium supplemented with 1 g of glucose and 100 mg of pyruvate/ml, 10% fetal calf serum, 2 mM glutamine, and antibiotics as described above. The cells were then seeded onto 24-well tissue culture dishes (Falcon, Lincoln Park, NJ) previously coated with an extracellular matrix produced by bovine corneal endothelial cells (19). Two days later the media were replaced with Dulbecco's modified Eagle's medium media supplemented as described above but containing 10% serum substitute (20) rather than 10% fetal calf serum. The cells were maintained in this medium for 2 days before their challenge with the congener being studied.

Each challenge consisted of a 1-hr preincubation in Dulbecco's modified Eagle's medium/10% serum substitute media containing ascorbic acid (1 ng/ml), bovine serum albumin (5 mg/ml), and nialamide (4.5 μg/ml) followed by a 3-hr incubation in the above media containing the various concentrations of DA congener (10^{-5} M to 10^{-11} M final concentration) being studied.

The amounts of PRL released by these cells in response to the DA congener challenge were determined in duplicate by double antibody radioimmunoassay using bovine PRL (RP-1) and rabbit anti-ovine PRL (S-1) supplied by the National Institute of Arthritis, Metabolism, and Digestive Diseases Pituitary Hormone Program.

Results

As has been shown previously, a single class of high affinity (K_d = 0.16 ± 0.01 nM) and saturable (maximum binding, 316 ± 25 fmol/mg of protein) DA-binding sites was defined by Scatchard analysis (n = 4) of [^{3}H]SPIR binding to bovine AP membranes. The mean K_d of 0.16 nM was used in all further calculations to determine the inhibitory constants (K_i) for each DA congener in the [^{3}H]SPIR competition binding studies.

[^{3}H]SPIR competition curves for the A.1 and B.1 monohydroxy congeners and for the A.3 and B.3 dihydroxy congeners are shown in Fig. 2. The B.1 and B.3 congeners (β-rotamers) competed for specific [^{3}H]SPIR-binding sites in bovine AP membranes in a biphasic manner. The biphasic curves were resolved into statistically discriminable high and low affinity binding sites ($p < 0.01$; Table 1). These two congeners recognized an equal number of specific [^{3}H]SPIR-binding sites. However, the dihydroxy congener recognized 38% of these binding sites with the high affinity, whereas the monohydroxy congener bound to 15% of the sites with high affinity. In contrast, the α-rotamers A.1 and A.3 competed in a low affinity, monophasic manner for the same number of binding sites.

The β-rotameric forms of the cyanomethyl substituted congeners B.2 and B.4 also competed for specific [^{3}H]SPIR-binding sites in a biphasic manner, recognizing statistically discriminable high and low affinity binding sites ($p < 0.01$) (Table 1). These two congeners recognized an equal number of binding sites but, again, as with the methylthiomethyl substituted congeners, the proportion of high affinity sites recognized by the dihydroxy congener B.4 was greater than that recognized by the monohydroxy congener B.2, 40 and 24%, respectively. Again, the α-rotameric forms of the cyanomethyl substituted congeners competed for specific [^{3}H]SPIR-binding sites in a monophasic manner filling an equivalent number of low affinity binding sites.

In keeping with the previous reports on the binding properties of AP DA receptors, the biphasic binding curves of the β-rotameric congeners were converted to monophasic curves by the addition of guanine nucleotides. The B.4 congener, for example, recognized only a single population of low affinity [^{3}H]SPIR-binding sites in the presence of 100 μM Gpp(NH)p (Fig. 3). When the guanine nucleotide was absent from the incubation media, B.4 recognized both high and low affinity binding sites.

Fig. 4 shows the inhibitory effect of increasing concentrations of the methylthiomethyl congeners on PRL release during a 3-hr incubation of bovine AP cells. It can be seen that the B.3 and B.1 congeners, which recognized high affinity [^{3}H]SPIR-binding sites (38% and 15%, respectively) were approximately 100 and 10 times more potent, respectively, as inhibitors of PRL release compared to the A.3 and A.1 congeners, which recognized only a single class of low affinity binding sites. The PRL IC_{50}, or that concentration of congener giving half-maximal inhibition of the PRL release, is listed in Table 1 for each DA congener tested.

The PRL-inhibiting activity of the cyanomethyl substituted congeners paralleled that of the methylthiomethyl substituted congeners (Table 1). The β-rotameric forms B.4 and B.2, which recognized high affinity binding sites (40% and 24%, respectively), were again approximately 100 and 10 times more potent, respectively, in inhibiting PRL release than the α-rotameric congeners A.4 and A.2, which recognized only low affinity binding sites.

Although the potencies (IC_{50} values) of various congeners varied greatly, their efficacy or maximum response was equivalent. All congeners decreased PRL release by a uniform 60–70% when tested at their maximum effective concentrations.

Interestingly, it is also evident from Table 1 that the dihydroxy congeners B.4 and B.3 were each approximately 10-fold more potent inhibitors of PRL release than their monohydroxy congener counterparts B.2 and B.1, respectively.

Discussion

The interactions of a novel series of rigid DA congeners with the bovine AP D-2 DA receptors were investigated in this study. Using indirect radioligand binding methods and computer modeling to analyze data, it was found that the β-rotameric conformations of these congeners interacted with the receptor in an agonist-specific fashion, exhibiting heterogeneous competition curves. These were resolved into discriminable high and low affinity binding sites. In contrast, the α-rotameric conformations of these congeners interacted with the receptor in a homogeneous fashion, exhibiting monophasic competition curves in which only the low affinity binding site was observable. Additionally, the dihydroxy β-rotameric congeners recognized greater proportions of the high affinity binding sites than their monohydroxy counterparts. These diverse receptor interactions were then correlated with the potency of these congeners to inhibit PRL release from bovine AP cells. A primary interest in examining these correlations was to determine the significance of the recognition of the two affinity binding sites to the biological response, i.e., the inhibition of PRL release.

The IC_{50} values of the congeners to inhibit PRL release are

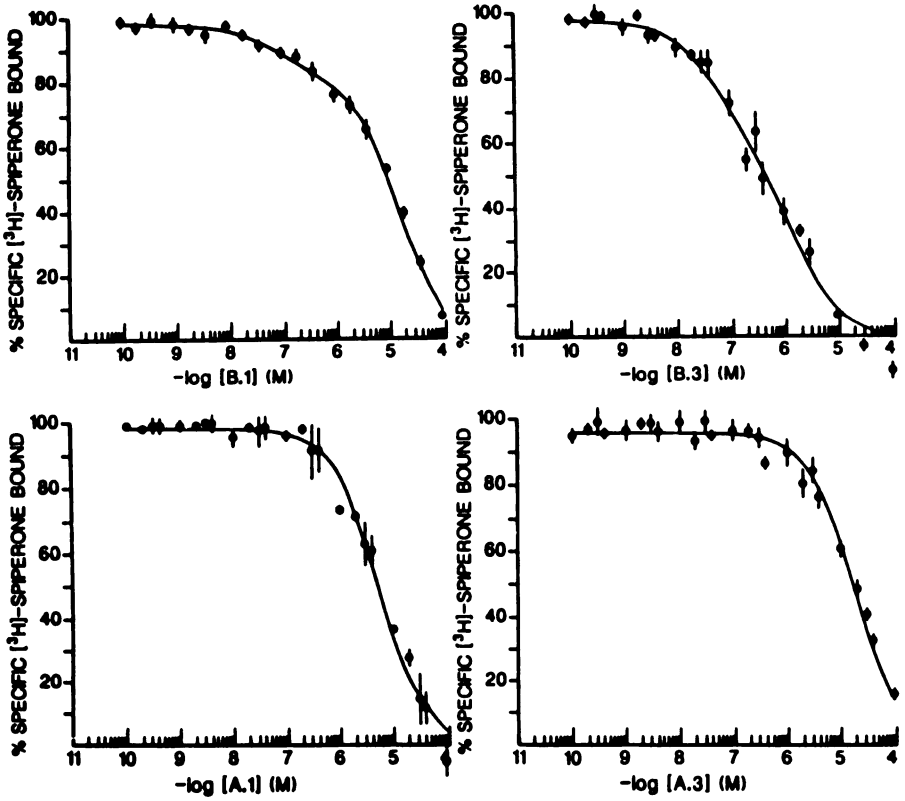

Fig. 2. [^{3}H]SPIR competition curves for the B.3 and A.3 dihydroxy congeners and the B.1 and A.1 monohydroxy congeners. Each *curve* depicts data pooled and analyzed from three individual competition binding assays. The *bars* through each *point* indicate the standard error for the three competitions performed. The B.3 and B.1 (β-rotamers) biphasic curves were resolved by computer-assisted analysis into statistically discriminable high and low affinity sites ($p < 0.01$). The A.3 and A.9 curves were monophasic and were resolved into a single population of low affinity sites.

TABLE 1

Computer-derived inhibition constants (K_i) at the two agonist-detected sites of the bovine anterior pituitary DA receptor ($K_{i\,low}$ and $K_{i\,high}$), proportions of [^{3}H]SPIR binding in high and low affinity sites recognized by the different DA congeners (% $K_{i\,high}$, % $K_{i\,low}$), and concentrations of the congeners to inhibit PRL secretion from bovine AP cells 50% of maximum (IC_{50})

Each value is the mean of triplicate determinations.

Congener	$K_{i\,low}$	%$K_{i\,low}$	$K_{i\,high}$	%$K_{i\,high}$	IC_{50}
	nM		nM		nM
A.1	286	100			119
A.3	1681	100			512
B.1	1234	85	6.6	15	26
B.3	129	62	4.9	38	0.6
A.2	4500	100			54
A.4	1474	100			62
B.2	259	76	1.8	24	6.4
B.4	312	60	6.4	40	0.5

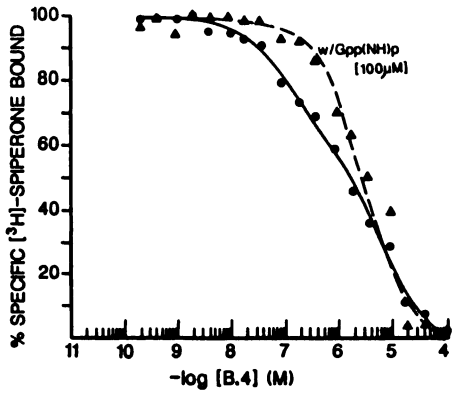

Fig. 3. [^{3}H]SPIR competition for B.4 in the absence (●) and presence (▲) of the nonhydrolyzable GTP analog Gpp(NH)p (100 μM final concentrations). In the presence of GPP(NH)p the characteristic biphasic curve of B.4 was converted to a monophasic curve which was resolved into a single population of low affinity sites.

correlated with their dissociation constants in Fig. 5. Considering first the β-rotameric congeners, which recognized both high and low affinity binding sites, it is evident that their IC_{50} values for inhibition of PRL release were more closely related to their inhibition constants for the high affinity site ($K_{i\,high}$). The $K_{i\,low}$ values for these congeners were between 1 and 3 orders of magnitude higher than the concentrations required to inhibit PRL release. This strongly suggests that the high affinity binding site of the AP D-2 DA receptor is the functionally important site mediating the inhibition of PRL release. These results corroborate those of DeLean *et al.* (21) and George *et al.* (10), who also found a correlation to exist between high affinity AP DA receptor binding and efficacy to inhibit PRL release. Considering next the α-rotameric congeners exhibiting monophasic low affinity [^{3}H]SPIR competition curves, their IC_{50} values for PRL inhibition were correlated with the $K_{i\,low}$ values. It seems unlikely that the PRL inhibition via these α-rotameric congeners is mediated through a receptor site distinct from that mediating the inhibition of PRL release by the β-rotameric congeners. It is likely that the α-rotameric congeners are working through the same receptor sites but recognize these sites with the same affinity (i.e. $K_{i\,high} = K_{i\,low}$). This is consistent with the observation that the α- and β-rotamers maximally suppressed PRL secretion to the same degree. Furthermore, the α-rotameric congeners displaced the same percentage of bound [^{3}H]SPIR as did the β-rotameric forms. A non-dopaminergic interaction can be excluded based upon the low potencies with which α- and β-adrenergic and serotonergic drugs compete for [^{3}H]SPIR-binding in the bovine AP (1, 22).

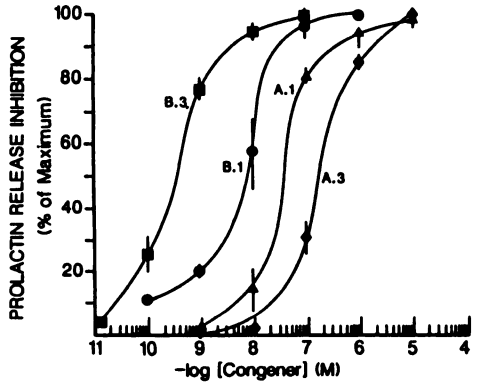

Fig. 4. Inhibitory effect of increasing concentrations of B.3, B.1, A.3 and A.1 on PRL release during a 3-hr incubation of bovine AP cells. The data are expressed as a percentage of the maximum PRL inhibition observed for each congener when tested at its maximum effective concentration. Each *point* and *bar* represent the mean ± standard error of the data pooled from the three individual experiments for each congener.

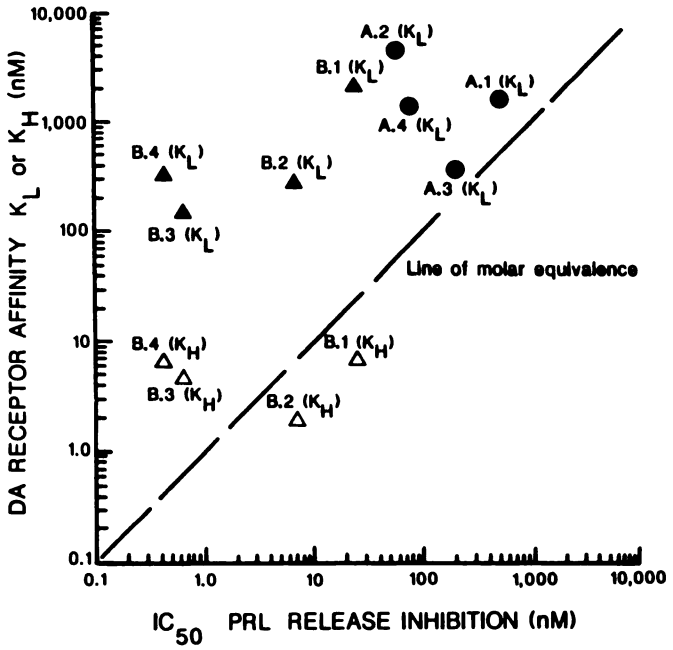

Fig. 5. Correlation of the IC_{50} values of the DA congeners to inhibit PRL release and the dissociation constants of the congeners at the high (K_H) and/or low (K_L) affinity agonist-binding sites of the DA receptor in bovine AP cells and membranes, respectively.

It can be observed (Fig. 5) that the receptor affinities and IC_{50} values for PRL inhibition were not equivalent (i.e., did not fall on the line of equivalent molarity). In partial explanation of this disparity, it is evident from the results of this study that the potency of this series of congeners is correlated not only with receptor site affinity but also with the relative proportion of the two affinity sites recognized by the congeners. The dihydroxy β-rotameric congeners, B.4 and B.3, recognized 40 and 38% of their binding sites with high affinity. Their monohydroxy counterparts B.2 and B.1 recognize only 24 and 15% of high affinity sites, respectively. The dihydroxy compounds were both approximately 10-fold more potent as inhibitors of PRL release than the monohydroxy compounds. This increased potency by the addition of a second hydroxyl group is not unique to this series of congeners but likewise enhances potency in other series of DA agonists. For example, (−)-*N*-*n*-propylnorapomorphine, with its two hydroxyls, is approximately 5–10 times more potent than its monohydroxyl counterpart (±)-11-hydroxy-*N*-*n*-propylnoraporphine (23). For many DA agonists a hydrogen-bonding group, frequently a hydroxyl group (at position 3 in DA), is essential for its interaction with its receptor (24, 25). It appears in this series of congeners that a second hydroxyl group, although not essential for dopaminergic activity, increases the ability of the congener to recognize high affinity DA-binding sites and thereby increases its potency.

The fraction of high affinity DA receptor sites is positively correlated with the inhibition of AP adenylate cyclase activity (15). A positive correlation between the degree of agonism and fraction of high affinity binding sites has been clearly demonstrated in the adenylate cyclase-coupled β-adrenergic receptor (26, 27). A ternary complex model involving guanine nucleotide-binding N proteins has been proposed to link high affinity agonist binding with receptor-mediated function in both the β-adrenergic (26–28) and AP DA receptor (5, 21).

A stumbling block in the acceptance of a functional relationship between high affinity DA agonist binding and PRL release inhibition is the finding that a high affinity DA agonist receptor-binding site is not identifiable on intact AP cells (29). In keeping with this finding, B.4, the congener that recognized the highest proportion of high affinity binding sites, competed for specific [^{3}H]SPIR-binding sites on dispersed, intact, bovine AP cells in a monophasic manner. The agonist affinity corresponded to the low affinity binding site observed in the AP cell membrane preparations ($K_{i_{low}}$ = 215 nM, data not shown). There is a plausable explanation for the inability to identify a high affinity agonist-binding site on intact AP cells. In equilibrium radioligand binding studies on intact cells, the endogenous guanine nucleotides would be predicted to rapidly and continuously convert the high affinity DA binding sites to those of low affinity. Guanine nucleotides have been shown to induce a complete conversion of high affinity DA-binding sites to those of low affinity in AP membranes (5, 6). In this study, Gpp(NH)p, a nonhydrolyzable GTP analog, converted the biphasic competition curves of the β-rotemeric congeners to monophasic low affinity curves.

In conclusion, the results of studies with this novel series of DA agonists have demonstrated that a correlation exists with both the affinity for, and the proportion of the high affinity agonist-binding site of the DA receptor recognized with potency to inhibit PRL release. In light of the nM concentrations of DA in the hypophysial blood reaching the AP, the high affinity binding state of the DA receptor appears to be the physiologically relevant conformation of the DA receptor mediating the inhibition of PRL release. Furthermore, the β-rotameric forms of these congeners induced or recognized the high affinity state of the receptor and were more potent PRL inhibitors than the α-rotameric congeners which demonstrated only low affinity binding. This result leads to the speculation that the AP DA receptor responds preferentially to the β-rotameric conformation of the flexible DA molecule.

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