0026-895X/88/010029-08$02.00/0
Copyright © by The American Society for Pharmacology and Experimental Therapeutics
All rights of reproduction in any form reserved.
MOLECULAR PHARMACOLOGY, 34:29-36

MOL PHARM

Dopamine D_1 Receptors of the Calf Parathyroid Gland: Identification and Characterization

H. B. NIZNIK, E. L. FOGEL, C. J. CHEN, D. CONGO, E. M. BROWN, AND P. SEEMAN

Departments of Medicine (H.B.N.) and Pharmacology (E.L.F., P.S.), University of Toronto, Toronto, Ontario, Canada M5S-1A8; and the Endocrine-Hypertension Unit, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115 (C.J.C., D.C., E.M.B.)

Received January 15, 1988; Accepted April 18, 1988

SUMMARY

The dopamine D_1 receptor was identified in the calf parathyroid gland. The binding of the selective D_1 receptor antagonist [^{3}H]SCH-23390 to membranes of calf parathyroid was specific, reversible, and saturable with a dissociation constant of ~200 pM and a receptor density of 30 fmol/mg of protein. Dopaminergic agonists and antagonists inhibited [^{3}H]SCH-23390 binding in a concentration-dependent and stereoselective manner with an appropriate pharmacological specificity for D_1 dopamine receptors. Moreover, potent dopaminergic agonists recognized two affinity forms of the receptor, one displaying high affinity for agonists, termed D_1^{High}, and one with low affinity, D_1^{Low}. The addition of the nonhydrolyzable guanine nucleotide guanyl 5′-yl-imidodiphosphate caused the complete transition of the agonist high affinity form (D_1^{High}) of the receptor to one displaying only low affinity for agonists (D_1^{Low}). Sodium ions, however, caused a ~5-fold decrease in the affinity of agonists at both D_1^{High} and D_1^{Low}. Virtually identical results were obtained on D_1 receptor preparations of neural origin. The D_1 receptor identified here appears to be the one responsible for the physiological effects on the parathyroid gland, because dopamine-stimulated cAMP accumulation is stereoselectively blocked by the D_1 receptor antagonist SCH-23390 in dispersed cells of the parathyroid gland. Moreover, a series of nine dopaminergic antagonists and agonists shows an excellent correlation between their potency in [^{3}H]SCH-23390 binding assays and their corresponding effects on cAMP accumulation. In the case of agonists, K_a for activation of cAMP accumulation agrees most closely with the agonist low affinity site in binding experiments. Specific [^{3}H]spiperone binding to D_2 dopamine receptors was not detected in this tissue and as such, the calf-parathyroid gland provides a model system in which to study the molecular characteristics of dopamine D_1 receptor-mediated events.

Two distinct receptor proteins for dopamine have been identified on the basis of biochemical and pharmacological criteria (see Refs. 1–5 for reviews). These receptors have been termed D_1 and D_2 and are classically defined by their ability to stimulate (D_1) or inhibit (D_2) adenylate cyclase activity and by their ability to respond to and bind specific dopaminergic agonists and antagonists.

As originally proposed by Kebabian and Calne (1), the bovine parathyroid gland contains the prototypical D_1 dopamine receptor. Thus, dopamine as well as other dopaminergic agonists including (±)-ADTN and the selective D_1 receptor agonist SKF-38393 stimulate the enzyme activity of adenylate cyclase, in a guanine nucleotide-dependent manner, in dispersed cells of the bovine parathyroid gland (6–9). Similarly, dopamine and dopaminergic congeners cause a transient increase of bovine immunoreactive PTH release both *in vitro* (7–10) and *in vivo* (11, 12). The stimulatory effects of dopamine in the parathyroid gland are independent of those produced by β-adrenergic agonist or low extracellular Ca^{2+} (13, 14) but are stereoselectively blocked by the nonselective dopamine receptor antagonist α-flupenthixol and not (or with much lower potency) by selective D_2 receptor antagonists such as sulpiride and YM-09151-2. Dopamine-mediated PTH release appears to be triggered by cAMP because agents known to mimic or increase cAMP (β-receptor stimulation, cholera toxin, dibutyryl cAMP, and phosphodiesterase inhibitors) increase PTH release (14–17), possibly by activating cAMP-dependent protein kinase activity and protein phosphorylation (18, 19).

Although these data are suggestive of a D_1 dopamine receptor, to date there have been no published reports describing the existence of a D_1 dopamine receptor in the bovine parathyroid gland by radioligand binding techniques. In this communication

H. B. N. is a recipient of a post-doctoral fellowship from the Medical Research Council of Canada. This work was supported by a grant from the Medical Research Council of Canada.

ABBREVIATIONS: (±)-ADTN; (±)-6,7,dihydroxy-2-aminotetralin; Gpp(NH)p, guanyl 5′-yl-imidodiphosphate; G_s, guanine nucleotide stimulatory binding protein; NPA, *N*-propylnorapomorphine; PTH, parathyroid hormone; SCH-23390, (*R*)-(+)-8-chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1*H*-3-benzazepine-7-ol; SKF-38393, 2,3,4,5-tetrahydro-7,8,dihydroxy-1-phenyl-1*H*-3-benzazepine; SKF-82526, 6-chloro-2,3,4,5-tetrahydro-1-(*p*-hydroxyphenyl)-1*H*-3-benzazepine-7,8-diol; LY-171555, (−)-(4a*R*,8a*R*)-4,4a,6,7,7,8,8a,9-octahydro-5-propyl-1*H*-pyrazolo[3,4-*g*]quinoline monohydrochloride; df, degrees of freedom; YM-09151-2, (*cis*)-*N*-1-benzl-2-methylpyrrolioin-3-yl)-5-chloro-2-methoxy-*u*-methylaminobenzamide.

MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 4, 2012

we document that a) membranes of calf parathyroid glands bind the selective D_1 receptor antagonist [^{3}H]SCH-23390 with high affinity and with a pharmacological profile indicative of a D_1 receptor subtype and b) dopamine-induced cAMP accumulation is mediated via the D_1 receptor.

Experimental Procedures

Materials. [^{3}H]SCH-23390 (73.4-85 Ci/mmol) and [^{3}H]spiperone (85 Ci/mmol) were obtained from Amersham (Oakville, Ontario, Canada). (±)-ADTN, (−)-butaclamol, and (−)-*N*-propylnorapomorphine were purchased from Research Biochemicals, Inc. (Wayland, MA). Dopamine, noradrenaline, serotonin, bromocriptine, and Gpp(NH)p were from Sigma Chemical Co. (St. Louis, MO). The following drugs were generously donated: (+)-butaclamol (Ayerst Research Laboratories, Montreal, Quebec, Canada); phentolamine (CIBA-GEIGY Corp., Dorval, Quebec); haloperidol, spiperone, ketanserin (Janssen Pharmaceutica, Beerse, Belgium); LY-171555 (Eli Lilly and Co., Indianapolis, IN); α-flupenthixol (H. Lundbeck and Co., Copenhagen, Denmark); (+)- and (−)-sulpiride (Ravizza, Milan, Italy); SCH-23390 and SCH-23388 (Schering Corp., Bloomfield, NJ); SKF-38393 and (*R*)- and (*S*)-SKF-82526 (Smith Kline and French, Philadelphia, PA).

Membrane preparation. Neonatal calf parathyroid glands were obtained from a local slaughterhouse (Boston, MA) by Dr. E. M. Brown, and canine brains were purchased from Pel Freez Biologicals, (Rogers, AR). Tissue was stored frozen at −70° until use. Parathyroid glands were thawed, trimmed of excess fat, weighed, minced, and immediately homogenized (Polytron, setting 6 for 20 sec; Brinkman, Inc., Westbury, NY) in 20 volumes of ice-cold 50 mM Tris·HCl buffer containing 5 mM EDTA, 1.5 mM $CaCl_2$, 5 mM $MgCl_2$, 5 mM KCl, and 120 mM NaCl (where indicated), pH 7.4 at 4°. Homogenates were filtered through four layers of cheesecloth and centrifuged for 15 min at 39,000 × *g*. The resulting pellet was resuspended in an original volume of buffer and recentrifuged. Striata were dissected from partially thawed brains, homogenized, and washed as above. Membrane pellets were resuspended in buffer to yield a final tissue concentration of 26 mg/ml original wet weight (parathyroid) or 4.5 mg/ml (brain) and rehomogenized (Polytron) for an additional 5 sec.

^{3}H-Ligand Binding assays. For saturation experiments, 0.5-ml aliquots of tissue homogenate (corresponding to 200 μg of protein) were incubated in triplicate with increasing concentrations of [^{3}H]SCH-23390 (10–4000 pM, final concentration) for 120 min at 22° in a total vol of 1.5 ml of 50 mM Tris·HCl buffer as described above (pH 7.4 at 22°) in either the presence or absence of 120 mM NaCl or 150 μM Gpp(NH)p. Incubations were terminated by rapid filtration through a Titertek Cell harvester (12 well; Skatron, Inc., Sterling, VA) using glass fiber "receptor binding" filter mats (Skatron). Filters were washed for 20 sec (~10 ml) with 50 mM Tris·HCl buffer (pH 7.4), placed in plastic mini-scintillation vials with 4 ml of Ready Solv EP (Beckman, Fullerton, CA) or Insta-Gel (United Technologies Packard, Downers Grove, IL) and monitored for tritium 16 hr later in a Packard 4660 liquid scintillation counter at ~34% efficiency.

For competition experiments, assays were initiated by the addition of 0.5 ml of membrane homogenate and incubated in duplicate with increasing concentrations of dopaminergic ligands (10^{-14}–10^{-3} M) and 0.25 ml of [^{3}H]SCH-23390 (150–200 pM final concentration) in the absence or presence of 120 mM NaCl, 150 μM Gpp(NH)p, or both, for 120 min at 22° in a final volume of 1.5 ml of Tris·HCl buffer (as above) containing 0.1% ascorbate and 12 μM nialamide (pH 7.4). Reactions were terminated by rapid vacuum filtration as described above.

For all experiments specific [^{3}H]SCH-23390 binding was defined as that inhibited by 1 μM (+)-butaclamol. This value was taken directly from (+)-butaclamol/[^{3}H]SCH-23390 competition curves, which indicate that at this concentration the binding of [^{3}H]SCH-23390 was suppressed to ~5% of control.

Data analysis. Both saturation and competition binding data were analyzed by the nonlinear least square, curve fitting program LIGAND, run on a Digital Micro-PDP-11 as previously described (20, 21).

cAMP accumulation in intact parathyroid cells. Dispersed bovine parathyroid cells were prepared as described previously (22) by digestion of minced neonatal parathyroid tissue with collagenase (1 mg/ml) and DNase (50 μg/ml). The final preparation of cells contained 90–95% parathyroid cells by light microscopy. Cell viability was routinely 95–100% as determined by trypan blue exclusion. No fat cells were detectable in preparations of this type by Sudan staining or cytocentrifuge preparations. Before incubations, cell counts were performed with a hemocytometer.

Incubations of cells were carried out with a final volume of 0.2 ml in 5-ml disposable scintillation vials (Sarstedt, Princeton, NJ) in a shaking water bath (Dubnoff-Precision Scientific Instruments, Sudbury, MA) at 37°. Standard medium for such incubations consisted of Minimal Essential Medium (Earle's salts with $NaHCO_3$, $CaCl_2$, and $MgSO_4$ deleted) with 0.02 M Hepes, pH 7.47, 2 mg/ml bovine serum albumin, 1.0 mM $CaCl_2$, 0.5 mM $MgSO_4$, and other additives as detailed below. At the end of the experiment, cAMP in cell pellets was extracted with 10% trichloroacetic acid. Residual trichloroacetic acid was removed by repeated extraction with diethylether (18). Radioimmunoassay for cAMP was carried out with a modification (14) of the method of Harper and Brooker (23). K_i values for dopaminergic antagonists were calculated from the relationship $k_i = IC_{50}/(1 + [S]/K_a)$, where IC_{50} is the concentration of antagonist half-maximally inhibiting the response due to a concentration [S] of dopamine (9). K_a is the concentration of dopamine producing half of the maximal increase in cAMP accumulation.

Protein determinations. Protein concentrations were determined by the bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, IL) as described by Smith *et al.* (24).

Results

Kinetics of [^{3}H]SCH-23390 binding. [^{3}H] SCH-23390 bound rapidly and reversibly to membranes of the calf parathyroid gland. Association experiments revealed that [^{3}H]SCH-23390 (100 pM, final concentration) binding reached equilibrium within 120 min at 22° as illustrated in Fig. 1. The association rate constant (K_{+1}) was determined to be 0.145 nM^{-1} min^{-1}. The dissociation rate constant of [^{3}H]SCH-23390 binding to parathyroid membranes was estimated (after equilibrium binding was achieved by the addition of 1 μM (+)-butaclamol) to be 0.0117 min^{-1} (Fig. 1, *inset*) with a corresponding half-life of dissociation ($t_{½} = 1n\ 2/K_{-1}$) of approximately 59 min. The K_D value obtained from these experiments (K_{-1}/K_{+1}) was 81 pM and agreed well with observed K_D values obtained directly from [^{3}H]SCH-23390 saturation experiments (see Fig. 2 and Table 1) or from SCH-23390 competition data (Fig. 3, Table 2). For striatal membranes, the association and dissociation rate constants for [^{3}H]SCH-23390 were (K_{+1}) 0.120 nM^{-1} min^{-1} and (K_{-1}) 0.0066 min^{-1}, with an estimated K_D of ~40 pM (data not shown).

Saturable binding of [^{3}H]SCH-23390. The saturable binding of [^{3}H]SCH-23390 to membranes of the parathyroid is illustrated in Fig. 2. [^{3}H]SCH-23390 binding was specific and saturable and displayed, in the presence of 120 mM NaCl, a dissociation constant of ~100 pM. The ligand appears to bind to a homogeneous population of binding sites with a specific activity of 30 ± 2 fmol/mg of protein. In the absence of sodium chloride, the K_D for [^{3}H]SCH-23390 was increased ~2-fold to ~220 pM, with no concomitant reduction in receptor density. As outlined in Table 1, similar results were obtained with [^{3}H] SCH-23390 binding to striatal receptor preparations and are consistent with previous observations (see Ref. 21). The addi-

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 4, 2012

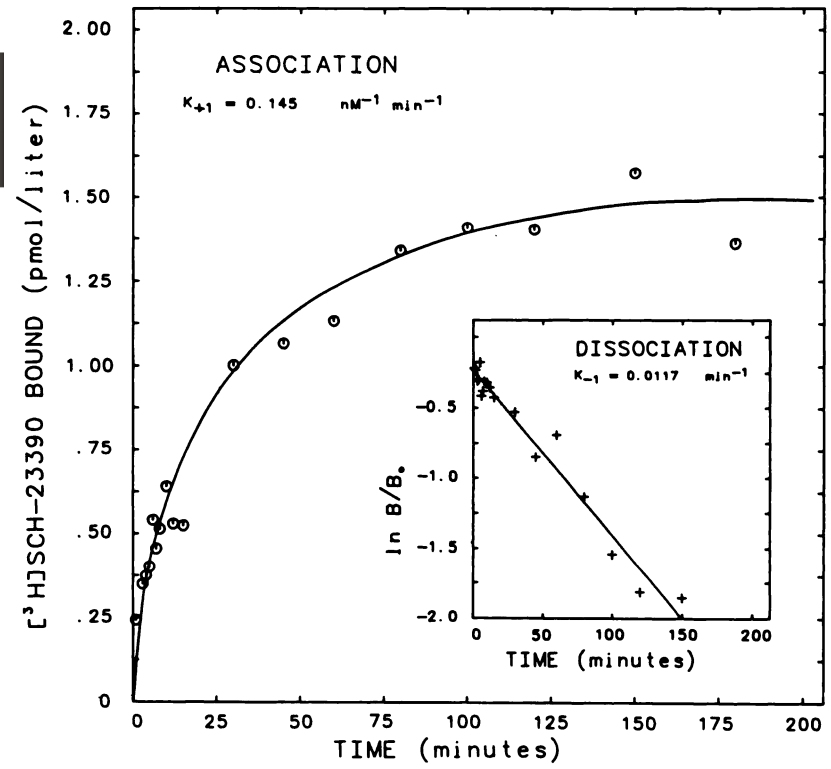

Fig. 1. Association and dissociation kinetics of [^{3}H]SCH-23390 binding to calf parathyroid membranes. For association experiments, parathyroid membranes (~150 μg of protein) were incubated with [^{3}H]SCH-23390 (100 pM) for various time periods. Nonspecific binding was defined by 1 μM (+)-butaclamol for each time point. The association rate constant (K_{+1}) for [^{3}H]SCH-23390 was determined (assuming pseudo-first order kinetics) by plotting [ln $B_e/(B_e - B)$] versus time, where B_e is the amount specifically bound (pM) at equilibrium and B is the amount bound at any given time point. The association rate constant was calculated from the following equation: [$K_{OB} - K_{-1} = K_{+1}(C_L)$], where K_{OB} is the slope of the association, K_{-1} is the dissociation rate constant, and C_L is the concentration of [^{3}H]SCH-23390 used. *Inset*, dissociation of [^{3}H]SCH-23390. After equilibrium, dissociation of [^{3}H]SCH-23390 was initiated by the addition of 1 μM (+)-butaclamol and assayed for D_1 receptor activity at various time periods. The dissociation rate constant was determined from the following equation: [ln $B/B_0 = K_{-1}\ t$] where B is the amount specifically bound, B_0 is the amount specifically bound at equilibrium, and t is time. Each *point* is the mean obtained from duplicate determinations and is representative of two independent observations with SE of <10%.

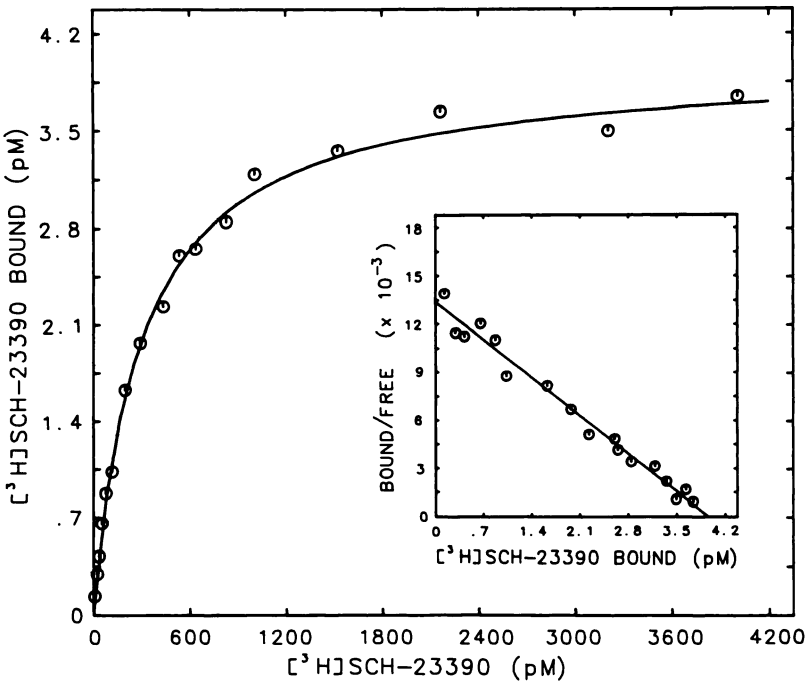

Fig. 2. Saturation of [^{3}H]SCH-23390 binding to membranes of the calf parathyroid. Parathyroid membranes (~200 μg of protein) were incubated for 120 min at 22° with increasing concentrations of [^{3}H]SCH-23390 (10–4000 pM), in a total assay volume of 1.5 ml, and assayed for D_1 receptor activity as described in Experimental Procedures. Nonspecific binding was defined by 1 μM (+)-butaclamol. The data were analyzed by the nonlinear, least square curve fitting program LIGAND as described. Each *point* represents the mean of duplicate determinations. B_{max} (pM) and (K_D) values for [^{3}H]SCH-23390 binding are listed in Table 1. *Inset*, Scatchard plot of the same data.

tion of the guanine nucleotide analog Gpp(NH)p did not significantly alter the K_D of [^{3}H]SCH-23390 binding to parathyroid or striatal membranes but did produce a slight, ~8–10%, increase in receptor density (see Table 1). The density of D_1 [^{3}H]SCH-23390 binding sites in the parathyroid gland is approximately 13-fold lower than that in canine striatal membranes. At saturation, nonspecific binding, as defined by (+)-butaclamol, represented from 40 to 50% of the total binding, whereas at lower ligand concentration (~150 pM), approximately 70% of the signal was specific.

Pharmacological specificity of [^{3}H]SCH-23390 binding. In order to establish whether calf parathyroid and striatal receptors display similar pharmacological specificity, the ability of various dopaminergic agonists and antagonists to compete for [^{3}H]SCH-23390 binding sites was investigated. Competition curves for [^{3}H]SCH-23390 binding to parathyroid membranes are illustrated in Fig. 3.

Dopaminergic agonists inhibited the specific binding of [^{3}H]SCH-23390 to calf parathyroid homogenates with the following rank order of potency: (*R*)-SKF-82526 (fenoldopam) > (−)-NPA > dopamine > (±)-ADTN > SKF-38393 > noradrenaline ≥ (*S*)-SKF-82526 ≫ (+)-NPA > serotonin > LY-171555 (quin-

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 4, 2012

TABLE 1

B_{max} and K_D values for [^{3}H]SCH-23390 binding to calf parathyroid and canine striatal membrane preparations

Calf parathyroid or canine striatal membranes (0.18 mg of protein) were incubated with increasing concentrations of [^{3}H]SCH-23390 (10–4000 pM) in the absence (control) or presence of 120 mM sodium or 150 μM Gpp(NH)p, as described in Experimental Procedures. Nonspecific binding was defined by 1 μM (+)-butaclamol. B_{max} (pM) and K_D (pM) values were estimated by LIGAND, as described. Values represent the means of three to six independent experiments with a SE of <15%.

Tissue/Condition	K_D	B_{max}	
	pM	pM[a]	fmol/mg of protein
Calf parathyroid			
Control	220 ± 16	3.6 ± 0.32	29.5 ± 2.7
Sodium	102 ± 12	3.6 ± 0.24	29.8 ± 1.9
Gpp(NH)p	235 ± 24	3.9 ± 0.43	32.0 ± 3.5
Canine striatum			
Control	149 ± 4.5	45.1 ± 0.9	376 ± 7.5
Sodium	86 ± 3.4	45.2 ± 0.9	377 ± 7.5
Gpp(NH)p	142 ± 7.1	48.6 ± 1.5	405 ± 12.2

[a] Units refer to picomoles of [^{3}H]SCH-23390 bound per liter of incubation medium.

pirole, a selective D_2 receptor agonist), clearly suggestive of a D_1 dopamine receptor profile. Similar results were obtained on the striatal D_1 receptor with the exception of noradrenaline, which displayed much lower affinity for D_1 sites in neural tissue than in the parathyroid gland (see Table 2).

Dopaminergic antagonists also inhibited [^{3}H]SCH-23390 binding in a concentration-dependent and stereoselective manner with the following order of potency: SCH-23390 > (+)-butaclamol > α-flupenthixol > SCH-23388 > haloperidol > spiperone > ketanserin. Moreover, the expected D_1 receptor selectivity for (+)- and (−)-sulpiride was seen, with (+)-sulpiride being more potent (Table 2). Selective D_2 receptor antagonists, such as eticlopride and raclopride, α-adrenergic antagonists, such as phentolamine and prazosin, or the inactive isomer of (+)-butaclamol were without effect on [^{3}H]SCH-23390 binding at concentrations of 10 μM or greater (data not shown).

As clearly depicted in Fig. 3, dopaminergic agonist/[^{3}H]SCH-23390 competition curves were shallow (i.e., a Hill slope of less than unity) with agonists displaying both high and low affinity for D_1 receptors of the parathyroid gland. Binding constants for each component of agonist/[^{3}H]SCH-23390 competition curves are listed in Table 2, in which D_1^{High} and D_1^{Low} represent binding of agonist to high and low affinity forms of the receptor. In addition, the relative proportions of receptors recognized by various dopaminergic agonists as existing in either the high or low affinity form are listed with corresponding values obtained from agonist/[^{3}H]SCH-23390 competition experiments conducted on canine striatal membranes.

Dopaminergic antagonist/[^{3}H]SCH-23390 competition curves were clearly monophasic (see Fig. 3) and best described as comprising a homogenous population of D_1 receptors. K_D values for antagonists at the D_1 receptor are listed in Table 2 along with K_D values obtained from striatal receptor preparations. As is evident from the data presented in Table 2 and graphically represented in Fig. 4, K_D values obtained for both agonists (D_1^{High}, D_1^{Low}) and antagonists at the D_1 receptor of the parathyroid gland correlate extremely well with agonist (high and low) and antagonist affinities for [^{3}H]SCH-23390 binding sites in striatal tissue. Taken together, these data are clearly suggestive that both the parathyroid and striatal D_1 dopamine receptor are pharmacologically homologous.

Modulation of agonist high affinity binding interactions. In order to determine whether the agonist high affinity form (D_1^{High}) of the D_1 receptor reflects the association of the receptor with a guanine nucleotide binding protein, presumably G_s, the ability of Gpp(NH)p to modulate the affinity of dopamine for the parathyroid D_1 receptor was investigated. As illustrated in Fig. 5A, dopamine (control) competed for [^{3}H]SCH-23390 binding in a biphasic manner ($F = 113.75$; df, 25,23, $p < 0.01$) with observed dissociation constants for D_1^{High} and D_1^{Low} of ~1.0 nM and 180 nM, respectively. Of the total receptor population labeled, dopamine recognized ~50% in the high affinity form and 50% in the low affinity form. The addition of the guanine nucleotide analog, Gpp(NH)p (150 μM), caused the complete transition of the agonist high affinity form of the receptor to one displaying only low affinity (~150 nM) for agonists, as a two-site fit was rejected ($F = 0.11$; df, 25,23, $p > .05$).

The addition of sodium ions, however (Fig. 5B), resulted in a significant 5–6-fold increase in the K_D for dopamine at both D_1^{High} and D_1^{Low}, as judged by the simultaneous analysis of control and NaCl-treated competition curves ($F = 160.91$; df, 24,23, $p < .01$) with no concomitant alteration in the proportion of receptors existing as either D_1^{High} or D_1^{Low} ($F = 0.59$, df, 24,23, $p > .05$; see Table 3). Furthermore, the addition of both Gpp(NH)p and sodium ions caused the complete conversion of the agonist high affinity form of the receptor to one displaying low affinity for dopamine, albeit with a 5-fold increase in the K_D of D_1^{Low}, to ~900 nM. Virtually identical results were obtained for dopamine/[^{3}H]SCH-23390 competition experiments conducted in striatal membrane preparations (K_D values are listed in Table 3).

[^{3}H]Spiperone (1 nM, final concentration) binding to D_2 dopamine receptors could not be detected in membranes of the parathyroid gland when 10 μM (−)-sulpiride was used to define nonspecific binding.

Modulation of cAMP content by dopaminergic ligands in intact bovine parathyroid cells. The D_1 receptor is defined physiologically in terms of the stimulation of adenylate cyclase activity. If the dopamine receptor defined by [^{3}H]SCH-23390 binding to parathyroid cells is relevant to the physiologically defined D_1 receptor, the affinity of dopaminergic agonists and antagonists for these sites should correlate closely with their apparent potency in modulating cAMP content. Fig. 6 shows the inhibition of dopamine-stimulated cAMP by SCH-23390 and SCH-23388. The former is markedly more potent than the latter, with an IC_{50} that is 3 orders of magnitude lower (~10^{-10} M versus 10^{-7} M, respectively). As listed in Table 4, there is a strong correlation between K_D values measured by binding analysis and K_a or K_i values determined from activation or inhibition, respectively, of cAMP accumulation for a series of dopaminergic agonists and antagonists. Moreover, as depicted graphically in Fig. 7, there is an excellent correlation between the K_D and K_i for antagonists as well as between the K_D for the low affinity binding site and K_a for agonists. The K_a values for agonists, however, are 2–3 orders of magnitude higher than the K_D of the high affinity agonist binding sites (D_1^{High}).

Discussion

The recent availability of the selective D_1 receptor antagonist SCH-23390 and its radiolabeled analogs has made it possible to identify and characterize D_1 dopamine receptors in neural

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 4, 2012

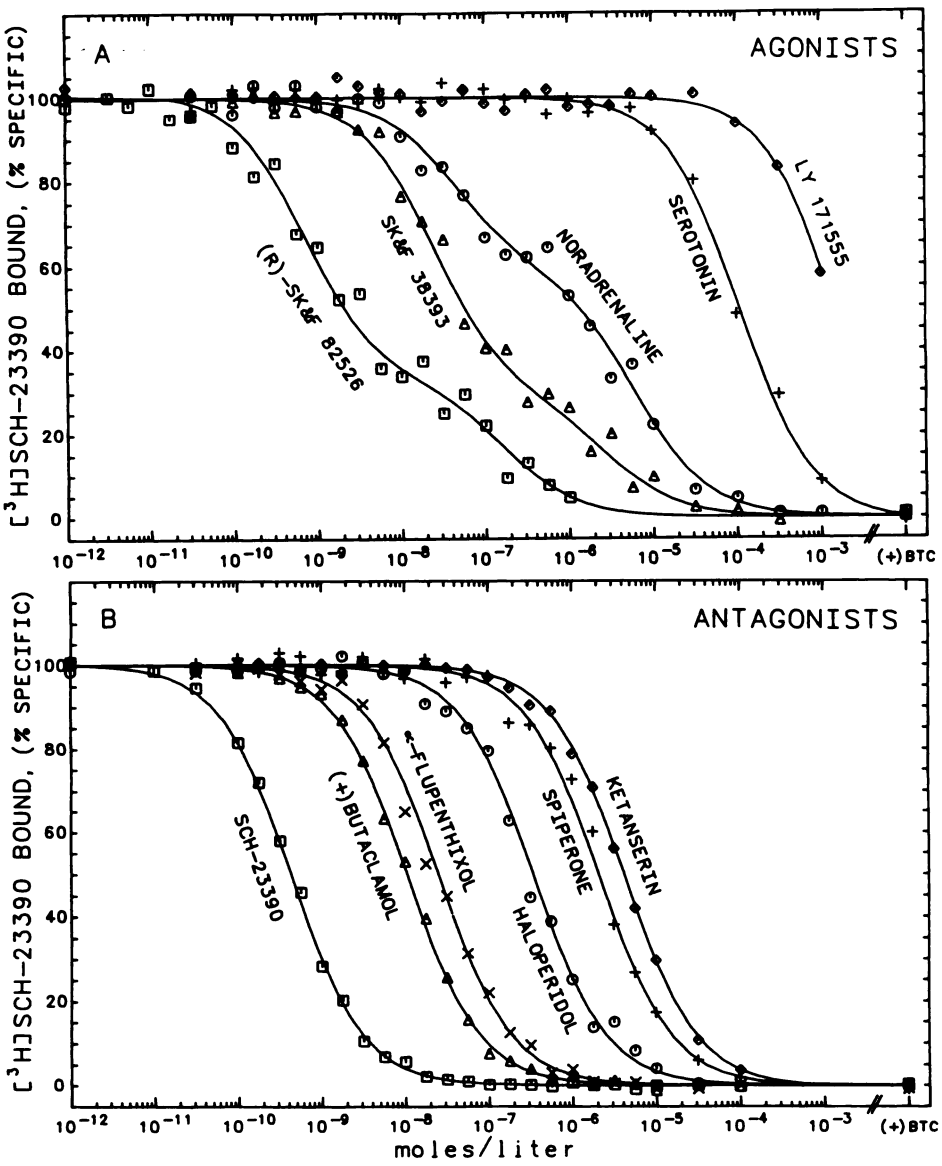

Fig. 3. Pharmacological specificity of [^{3}H] SCH-23390 binding to membranes of the calf parathyroid gland. Increasing concentrations of agonists and antagonists were incubated with membranes (~200 μg of protein) and [^{3}H]SCH-23390 (150–200 pM) for 120 min at 22° in sodium-free buffer for agonists or with sodium for antagonists. Nonspecific binding was defined in the presence of 1 μM (+)-butaclamol. Bound was separated from free [^{3}H]-ligand by vacuum filtration as described in Experimental Procedures. Data were analyzed by LIGAND for both one- and two-site fits. The results shown here are the means of triplicate determinations and are representative of two or three such experiments. Dissociation constants for agonists and antagonists under these experimental conditions are listed in Table 2.

tissue (reviewed in Refs. 4 and 5). In this communication, we show that the calf parathyroid gland contains the D_1 dopamine receptor, as indexed by the selective, reversible, saturable, high affinity binding of [^{3}H]SCH-23390. Moreover, dopaminergic agonists and antagonists compete for [^{3}H]SCH-23390 binding with a pharmacological profile suggestive of D_1 receptors. Thus, the benzazepines SKF-82526, SKF-38393, and SCH-23390 display high affinity for D_1 sites labeled by [^{3}H]SCH-23390 whereas selective D_2 receptor agonists (LY-171555) and antagonists (spiperone, eticlopride, and (−)-sulpiride) are virtually inactive. Furthermore, the data obtained in membranes of calf parathyroid gland compare favorably with those obtained in neural tissue (canine striatum) under identical assay conditions and are suggestive of the contention that the D_1 receptor in the parathyroid and brain share pharmacological homology.

Despite the strong pharmacological correlations seen between D_1 receptors in the parathyroid and brain, some discrepencies were noted. First, as outlined in Table 2 and in Fig. 4, virtually all dopaminergic agonists displayed a 2–10-fold higher affinity for D_1 receptors in the calf parathyroid than in canine striatum. Although species differences might account for these observations (see Refs. 21 and 25) it is interesting to note that the K_D values of agonists at D_1 receptors in the parathyroid gland are similar to those observed in digitonin-solubilized D_1 receptor preparations of *canine* striatal membranes (21) and this suggests that the removal of some endogenous factor, possibly dopamine, may influence the K_D of agonists at the D_1 receptor. It is of interest to note also that noradrenaline displays approximately 30-fold higher affinity for D_1 receptors in the parathyroid than in the brain.

As shown in Table 2, the relative proportions of the D_1^{High} and D_1^{Low} affinity forms of the receptor recognized by various dopaminergic agonists differed in both tissues. Whether this reflects an altered stoichiometry of D_1 receptor-G_s coupling, the product of D_1-D_2 receptor-effector cross-talk (in neural tissue), or the presence of endogenous dopamine is unknown at present. Furthermore, there appears to be no obvious correlation between the proportions of agonist high or low affinity forms of the receptor recognized by dopaminergic agonists and their ability to stimulate adenylate cyclase or PTH release. Thus, (±) -ADTN or dopamine, classified as full agonists, recognize (within ~10%) the same proportions of D_1 receptors existing

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 4, 2012

TABLE 2

Agonist and antagonist dissociation constants for calf parathyroid and canine striatal D_1 dopamine receptors

Membrane preparations (0.18 mg of protein) obtained from calf parathyroids or canine striata were incubated with varying concentrations of dopaminergic agonists (10^{-12}–10^{-3} M) or antagonists (10^{-14}–10^{-4} M) and ~150 pM [^{3}H]SCH-23390, as described in Experimental Procedures and in the legend to Fig. 3. All data were analyzed by LIGAND for both one- and two-site fits. Values represent the means of two or three independent experiments with a SE of <15%.

Agonists	K_D		Proportions	
	D_1^{High}	D_1^{Low}	D_1^{High}	D_1^{Low}
	nM		%	
Parathyroid				
(*R*)-SKF-82526	0.3	69	68	32
(−)-NPA	0.8	155	46	54
Dopamine	1.2	185	48	52
(±)-ADTN	3.9	500	60	40
SKF-38393	10.0	1,139	71	29
Noradrenaline	17.8	2,505	41	59
(*S*)-SKF-82526	20.4	1,114	53	47
(+)-NPA		868		–[a]
Bromocriptine	(−sodium)	1,154		–[a]
	(+sodium)	2,708		–[a]
Serotonin		46,000		–[a]
LY-171555		785,000		–[a]
Striatum				
(*R*)-SKF-82526	3.3	388	95	5
(−)-NPA	5.0	332	15	85
Dopamine	5.6	653	21	79
(±)-ADTN	20.4	1,266	48	52
SKF-38393	21.0	260	42	58
(*S*)-SKF-82526	90.0	910	28	72
Noradrenaline	561.0	12,980	27	73
Bromocriptine	(−sodium)	475		–[a]
	(+sodium)	778		–[a]
(+)-NPA		1,360		–[a]
Serotonin		31,000		–[a]
LY-171555		421,000		–[a]

Antagonists	K_D	
	Parathyroid	Striatum
	nM[a]	
SCH-23390	0.14	0.16
(+)-Butaclamol	4	4
Flupenthixol	7	7
SCH-23388	24	18
Haloperidol	102	73
Spiperone	627	415
Ketanserin	1,260	755
(*R*)-(+)-Sulpiride (+NaCl)	6,000	12,000
(−NaCl)	12,000	26,000
(*S*)-(−)-Sulpiride (+NaCl)	20,000	57,000
(−NaCl)	>100,000	>100,000

[a] Curves were uniphasic and could not be fit to two sites.

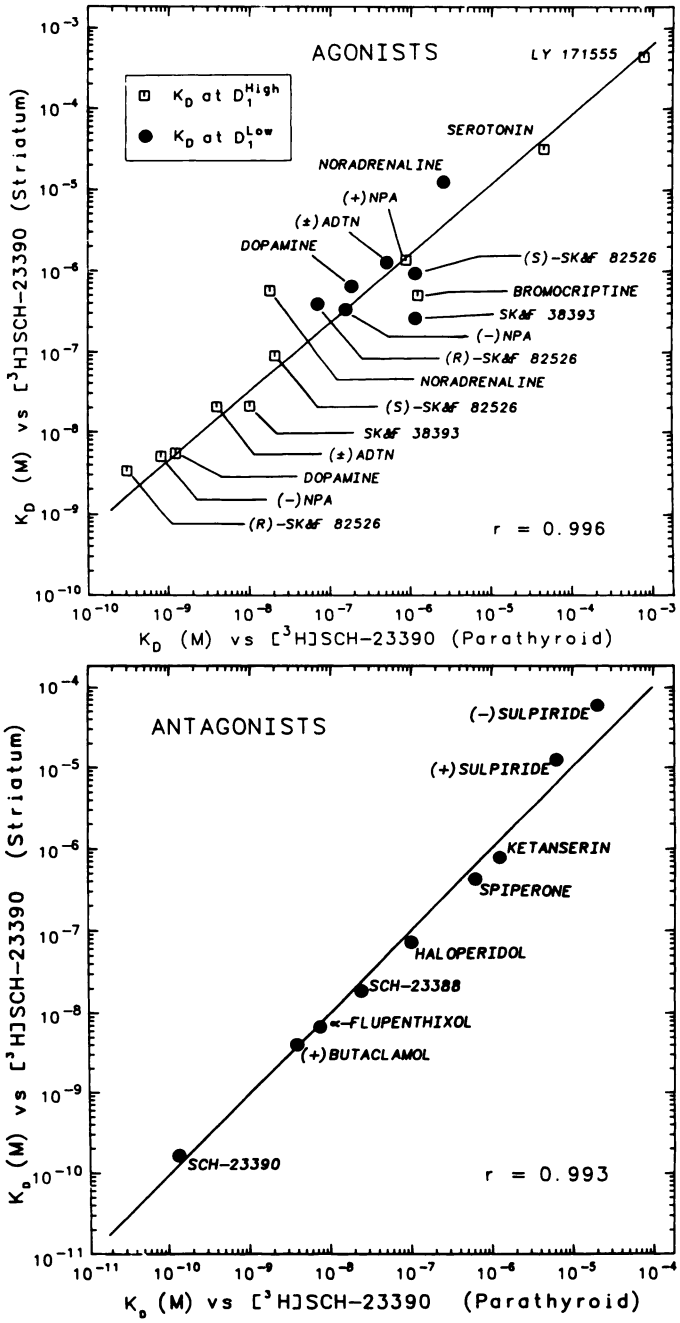

Fig. 4. Correlational plot of agonist and antagonist K_D values for D_1 receptors in the parathyroid gland and brain striata. K_D values for agonists and antagonists were derived from competition binding experiments as described in Fig. 3 and Table 2. The concentration of [^{3}H]SCH-23390 used was 150–200 pM.

as either in agonist high or low affinity as does SKF-38393, which is a partial agonist (8).

As with the β-adrenergic receptor, which is linked in a stimulatory fashion to adenylate cyclase, the association of D_1 receptors with a guanine nucleotide-binding protein, presumably G_s, has been implied by virtue of the fact that guanine nucleotides modulate agonist interactions with D_1 receptors in neural tissue and that dopamine-stimulated adenylate cyclase activity is GTP- and cholera-toxin sensitive (see Ref. 5 for references). Similarly, in the calf parathyroid gland, dopamine high affinity interactions with the D_1 receptor are modulated by Gpp(NH)p, presumably by uncoupling a D_1 receptor-G_s complex with a concomitant abolition of the agonist high affinity form (D_1^{High}) of the receptor (see Fig. 5). Although G_s is presumed to be coupled to D_1 receptors, the exact molecular form of this protein (of which there are at least two; see Ref. 26) is unknown. Sodium ions, however, do not cause the transition of the agonist high affinity form of the receptor to one displaying only low affinity but simply reduce the affinity for agonists at both D_1^{High} and D_1^{Low}. Whether the effects of sodium are mediated via a) a conformational change in the D_1 receptor,

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 4, 2012

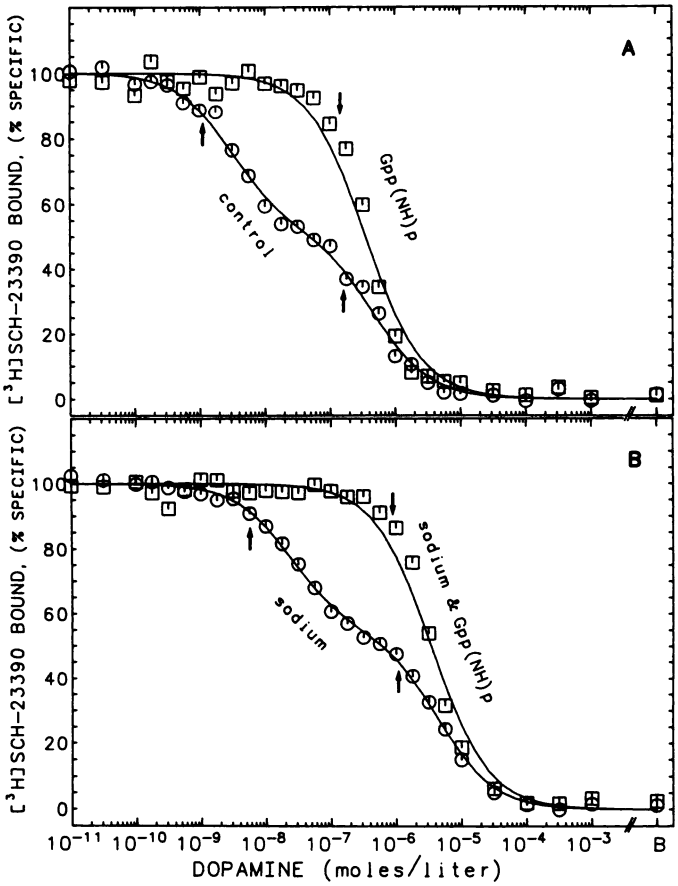

Fig. 5. Modulation of agonist interaction with parathyroid D_1 receptors by Gpp(NH)p and sodium chloride. Parathyroid membranes (180 μg of protein) were incubated with [^{3}H]SCH-23390 (~200 pM) and increasing concentrations of dopamine in the absence or presence of Gpp(NH)p (150 μM), 120 mM NaCl, or both for 120 min at 22° and assayed for D_1 receptor activity as described. The data were analyzed by LIGAND as described in Experimental Procedures. Dissociation constants of dopamine (indicated by *arrows*) for agonist high and low affinity forms of the D_1 receptor under these experimental conditions are listed in Table 3. The results shown here are from an experiment conducted in duplicate and are representative of two or three independent determinations. Results of the simultaneous analysis of control and experimental curves are described in the text.

b) direct effects on a guanine nucleotide-binding protein, or c) a product of ionic strength is unknown. In any event, these data suggest that the locus of action of Gpp(NH)p and NaCl to affect D_1 agonist high affinity binding interactions are probably not the same.

This report also documents that the D_1 dopamine receptor identified in the calf parathyroid gland by using [^{3}H]SCH-23390 is the same as the dopamine receptor mediating cAMP accumulation in this tissue. Thus, dopamine-stimulated cAMP accumulation in dispersed parathyroid cells was stereoselectively inhibited by the selective D_1 receptor antagonist SCH-23390. Moreover, the potencies of several dopaminergic antagonists agree well with their K_D values for [^{3}H]SCH-23390 binding sites. In the case for agonists, K_a values for cAMP accumulation agree best with the K_D values for the D_1^{Low} affinity form of the D_1 receptor (see Fig. 7). It is possible that in the intact cell the affinity of D_1^{High} is reduced by high ambient concentrations of NaCl such that it is closer to the values for D_1^{Low} in membrane preparations (see Table 3). Alternatively,

TABLE 3

Regulation of agonist affinity for calf parathyroid and canine striatal D_1 dopamine receptors

Calf parathyroid or canine striatal membranes were incubated with 10^{-12}–10^{-3} M dopamine in the absence or presence of 120 mM sodium chloride, 150 μM Gpp(NH)p, or both and 150–200 pM [^{3}H]SCH-23390 for 120 min at 22°, as described in Experimental Procedures. Nonspecific binding was defined by 1 μM (+)-butaclamol. Residual free [^{3}H]SCH-23390 was separated from bound by rapid vacuum filtration. Data were analyzed by computer for both one- and two-site fits. Significant changes in estimated parameters of control versus experimental competition data were determined by simultaneous analysis as described in the text. Values are means of two or three independent experiments with a SE of <15%.

	K_D of Dopamine		Proportions	
	D_1^{High}	D_1^{Low}	D_1^{High}	D_1^{Low}
	nM		%	
Calf parathyroid				
Control	1.0	181	48	52
Sodium	6.0*	1088*	45	55
Gpp(NH)p		141	0*	100
Sodium and Gpp(NH)p		872*	0*	100
Canine striatum				
Control	5.5	653	21	79
Sodium	69.5*	1900*	28	72
Gpp(NH)p		709	0*	100
Sodium and Gpp(NH)		2224*	0*	100

* $p < 0.01$ with respect to control condition.

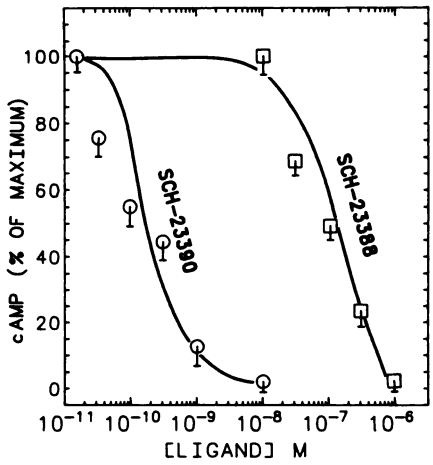

Fig. 6. Inhibition of dopamine-stimulated cAMP accumulation by SCH-23390 and SCH-23388 in intact bovine parathyroid cells. Dispersed parathyroid cells (2×10^6 ml) were incubated for 5 min at 37° with 10^{-6} M dopamine, the indicated concentrations of SCH-23390 or SCH-23388, 1.0 mM Ca^{2+}, and 0.5 mM Mg^{2+}. The reaction was terminated by the addition of 10% trichloroacetic acid (final v/v), and cAMP was determined as described in Experimental Procedures. Results are shown as the mean ± standard error for cAMP expressed as percentage of dopamine-stimulated cAMP in the absence of added antagonist (4–10 separate points in two or three experiments). With 10^{-6} M dopamine, cAMP was 1.18 ± .14 pmol/10^5 cells; without dopamine, cAMP was 0.033 ± .007 pmol/10^5 cells.

D_1^{High} may represent a form of the receptor that is not tightly coupled to adenylate cyclase or even a binding site that is not relevant to the biological activity of the physiologically defined D_1 receptor. Studies of [^{3}H]SCH-23390 binding to intact parathyroid cells and/or to permeabilized cells incubated with GppNHp might clarify this issue.

In neural tissue, D_1 receptors appear to allow for the expression of D_2 dopamine-mediated events at both the behavioral and neurophysiological level (27–29). The calf parathyroid gland does not appear to contain dopamine receptors of the D_2 subtype, inasmuch as no detectable specific binding of the D_2 receptor antagonist [^{3}H]spiperone was observed. The calf para-

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 4, 2012

TABLE 4

D_1 binding affinity versus cAMP accumulation

Agonist and antagonist K_D values for binding experiments with membranes versus K_a for K_i for effects on cAMP accumulation in intact cells. K_d values for agonists were derived from competition binding experiments as described in Fig. 3 and Table 2. Values for K_a or K_i were determined as outlined in Experimental Procedures. Each point represents results from two or more experiments. For agonists, values are shown for both D_1^{High} and D_1^{Low}.

Ligand	K_a or K_i, cAMP	K_D	
		D_1^{High}	D_1^{Low}
		μM	
(R)-SKF-82526	0.1	0.0003	0.070
(±)-ADTN	0.5	0.004	0.50
Dopamine	0.6	0.001	0.185
SKF-38393	1.0	0.01	1.100
SCH-23390	0.00006	0.00014	
(+)-Butaclamol	0.005	0.004	
α-fluphentixol	0.03	0.007	
SCH-23388	0.037	0.024	
(*R*)-(+)-Sulpiride	13	6	

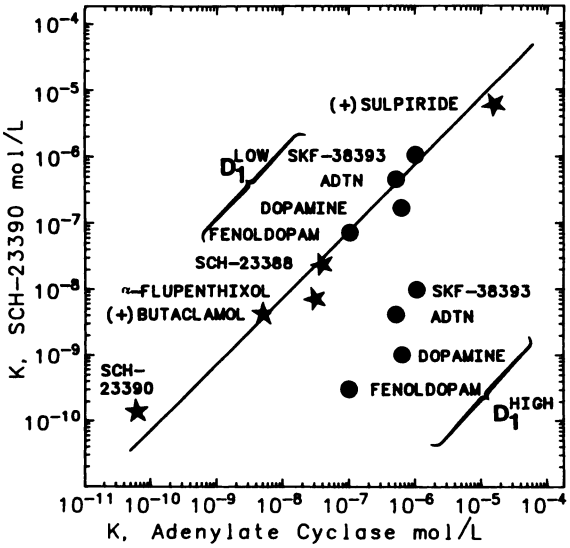

Fig. 7. Correlation plot of agonist and antagonist *K* values for cAMP accumulation and [^{3}H]SCH-23390 binding in calf parathyroid glands. K_a or K_i values for dopaminergic effects on cAMP production in intact cells of the bovine parthyroid gland were determined as described in Experimental Procedures and tabulated in Table 4. K_D values for agonists and antagonists were derived from competition binding experiments with [^{3}H] SCH-23390 as described in Fig. 3 and Table 2 (r = 0.991).

thyroid gland therefore provides a model system in which to study the functionality of the D_1 dopamine receptor at both the structural and molecular level, uninfluenced by and independent of the activity of the D_2 receptor system.

Acknowledgments

The authors would like to thank Anne Tirpak for technical assistance and Marie Botelho for typing the manuscript.

References

1. Kebabian, J. W., and D. B. Calne. Multiple receptors for dopamine. *Nature (Lond.)* **277**:93–96 (1979).
2. Seeman. P. Brain dopamine receptors. *Pharmacol. Rev.* **32**:229–313 (1980).
3. Stoof, J. C., and J. W. Kebabian. Two dopamine receptors: biochemistry, physiology and pharmacology. Life Sci. **35**:2281–2296 (1984).
4. Seeman, P., and D. E. Grigoriadis. Dopamine receptors in brain and periphery. *Neurochem. Int.* **10**:1–25 (1987).
5. Niznik, H. B. Dopamine receptors: molecular structure and function. *Mol. Cell. Endocrinol.* **54**:1–22 (1987).
6. Brown, E. M., R. J. Carroll, and G. D. Aurbach. Dopaminergic stimulation of cyclic AMP accumulation and parathyroid hormone release from dispersed bovine parathyroid cells. *Proc. Natl. Acad. Sci. U.S.A.* **74**:4210–4213 (1977).
7. Attie, M. F., E. M. Brown, D. G. Gardner, A. M. Spiegel, and G. D. Aurbach. Characterization of dopamine responsive adenylate cyclase of bovine parathyroid cells and its relationship to parathyroid hormone secretion. *Endocrinology* **107**:1776–1781 (1980).
8. Brown, E. M., and B. Dawson-Hughes. D_1 Dopamine receptor-mediated activation of adenylate cyclase, cAMP accumulation, and PTH release in dispersed bovine parathyroid cells, in *Dopamine Receptors* (C. Kaiser, and J. W. Kebabian, eds.). American Chemical Society Symposium Series 224, Washington, D.C., 1–22 (1983).
9. Brown, E. M., M. F. Attie, S. Reen, D. G. Gardner, J. W. Kebabian, and G. D. Aurbach. Characterization of dopaminergic receptors in dispersed bovine parathyroid cells. *Mol. Pharmacol.* **18**:335–340 (1980).
10. Hanley, D. A., and P. G. Wellings. Dopamine stimulated parathyroid hormone release *in vitro*: further evidence for a two pool model of parathyroid secretion. *Can. J. Physiol. Pharmacol.* **63**:1139–1144 (1985).
11. Blum, J. W., P. Kunz, J. A. Fischer, U. Binswanger, W. Lichten Steiger, and M. Daprada. Parathyroid hormones response to dopamine in cattle. *Am. J. Physiol.* **239**:E255–E264 (1980).
12. Blum, J. W., and J. A. Fischer. Parathyroid hormone responses to dopaminergic agonist, (±)-ADTN. *Life Sci.* **35**:1469–1474 (1984).
13. Brown, E. M., R. Leombruno, J. Thatcher, and M. Burrowes. The acute secretory response to alterations in extracellular calcium concentration and dopamine in perfused bovine parathyroid cells. *Endocrinology* **116**:1123–1132 (1985).
14. Brown, E. M., S. Hurwitz, and G. D. Aurbach. Beta-adrenergic stimulation of cyclic AMP content and parathyroid hormone release from isolated bovine parathyroid cells. *Endocrinology* **100**:1696–1702 (1977).
15. Brown, E. M., D. G. Gardner, R. A. Windeck, and G. D. Aurbach. Cholera toxin stimulates 3′-5′ adenosine monophosphate accumulation and parathyroid hormone release from dispersed bovine parathyroid cells. *Endocrinology* **104**:218–225 (1979).
16. Brown, E. M., D. G. Gardner, R. A. Windeck, and G. D. Aurbach. Relationship of intracellular 3′-5′ adenosine monophosphate accumulation to parathyroid hormone release from dispersed bovine parathyroid cells. *Endocrinology* **103**:2323–2333 (1978).
17. Morrissey, J. J., and D. V. Cohn. Secretion and degradation of parathyroid hormone as a function of intracellular maturation of hormone pools. *J. Cell. Biol.* **83**:521–528 (1979).
18. Brown, E. M., and J. G. Thatcher. Adenosine 3′5′ monophosphate (cAMP) dependent protein kinase and the regulation of PTH release by divalent cations and agents elevating cAMP in dispersed bovine parathyroid cells. *Endocrinology* **110**:1374–1380 (1982).
19. Lasker, R. D., and A. M. Spiegel. Endogenous substrates for cAMP dependent phosphorylation in dispersed bovine parathyroid cells. *Endocrinology* **111**:1412–1414 (1982).
20. Grigoriadis, D., and P. Seeman. Complete conversion of brain D_2 dopamine receptors from the high-to-low affinity state for dopamine agonists, using sodium ions and guanine nucleotides. *J. Neurochem.* **44**:1925–1935 (1985).
21. Niznik, H. B., N. Otsuka, A. Dumbrille-Ross, D. Grigoriadis, A. Tirpak, and P. Seeman. Dopamine D_1 receptors characterized with [^{3}H]SCH-23390: solubilization of a guanine nucleotide sensitive form of the receptor. *J. Biol. Chem.* **261**:8397–8406 (1986).
22. Brown, E. M., S. Hurwitz, and G. D. Aurbach. Preparation of viable isolated bovine parathyroid cells. *Endocrinology* **99**:1582–1588 (1976).
23. Harper, J. F., and G. Brooker. Femtomole sensitive radioimmunoassay for cAMP and cGMP after 2′-*O*-acetylation by acetic anhydride in aqueous solution. *J. Cyclic Nucleotide Res.* **1**:207–218 (1975).
24. Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Malha, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**:76–85 (1985).
25. Seeman, P., and H. B Niznik. Pharmacology of dopamine D_1 receptors. *ISI Atlas of Sci.: Pharmacol.* **2**:161–170 (1988).
26. Spiegel, A. M. Signal transduction by guanine nucleotide binding proteins. *Mol. Cell. Endocrinology* **49**:1–16 (1987).
27. Waddington, J. L. Behavioural correlates of the action of selective D_1 dopamine receptor antagonists: impact of SCH-23390 and SKF-38393, and functionally interactive D_1:D_2 receptor systems. *Biochem. Pharmacol.* **35**:3661–3667 (1986).
28. Walters, J. R., D. A. Bergstrom, J. H. Carlson, T. N. Chase, and A. R Braun. D_1 dopamine receptor activation required for postsynaptic expression of D_2 agonist effects. *Science, (Wash. D.C.)* **236**:719–722 (1987).
29. Clark, D., and F. J. White. D_1 dopamine receptor: the search for a function. *Synapse (N. Y.)* **1**:347–388 (1987).

Send reprint requests to: H. B. Niznik, Ph.D., Department of Pharmacology, Medical Science Bldg., University of Toronto, Toronto, Ontario M5S 1A8, Canada.

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 4, 2012