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Differential Activation of Adenylate Cyclase and Receptor Internalization by Novel Dopamine D_1 Receptor Agonists

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ABSTRACT

Structurally dissimilar dopamine D_1 receptor agonists were compared with dopamine in their ability to activate adenylate cyclase and to internalize hemagglutinin-tagged human D_1 receptors in a stably transfected human embryonic kidney cell line. Thirteen dopamine D_1 receptor agonists were selected rationally from three different structural classes: rigid fused ring compounds [dihydrexidine, dinapsoline, dinoxyline, apomorphine, and (5*aR*,11*bS*)-4,5,5*a*,6,7,11*b*-hexahydro-2-propyl-3-thia-5-azacyclopent-1-ena[*c*]-phenanthrene-9,10-diol (A86929)]; isochromans [(1*R*,3*S*)-3-(1′adamantyl)-1-aminomethyl-3,4-dihydo-5,6-dihydroxy-1*H*-2-benzopyran (A77636) and (1*R*,3*S*)-3-phenyl-1-aminomethyl-3,4-dihydo-5,6-dihydroxy-1*H*-2-benzopyran (A68930)]; and benzazepines [7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine (SKF38393), (±)-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine (SKF77434), 6-chloro-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine (SKF82958), 3-methyl-6-chloro-7,8-hydroxy-1-[3-methylphenyl]-2,3,4,5-tetrahydro-]*H*-3-benzazepine (SKF83959), *R*(+)-6-chloro-7,8,-dihydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine (SKF82957), and *R*(+)-6-chloro-7,8,-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine (SKF81297)]. The working hypothesis was that some agonists have differential effects on adenylate cyclase versus receptor internalization that could be correlated to the structural class of the agonist. First, the affinity for the hemagglutinin-hD_1 receptor and the intrinsic activity and potency of adenylate cyclase activation were determined for each compound. The internalization time course and internalization efficacy were then determined for each agonist. It was surprising that internalization efficacy was found to be independent of either agonist structural class or affinity. Only agonists that had both high adenylate cyclase functional potency and high intrinsic activity caused internalization. In addition, four agonists from two structural classes were identified that were capable of fully activating adenylate cyclase without eliciting an internalization response. This study provides the first extensive characterization of D_1 receptor internalization in response to structurally diverse agonists and, at least for the D_1 receptor, shows that functional selectivity is not predictable by simple structural examination. These data are consistent with the hypothesis that functional selectivity reflects subtle ligand-induced conformational changes as opposed to simple agonist trafficking among discrete receptor active states.

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The dopamine receptors are a superfamily of heptahelical G protein-coupled receptors (GPCRs) that have historically been divided into "D_1-like" and "D_2-like" subfamilies (Garau et al., 1978; Kebabian and Calne, 1979). The dopamine D_1 receptor is a member of the "D_1-like" subfamily and couples to adenylate cyclase through the stimulatory G proteins G_s and G_{olf} (Herve et al., 1993). Dopamine D_1 receptor agonists that are similarly efficacious to dopamine in activating ade-

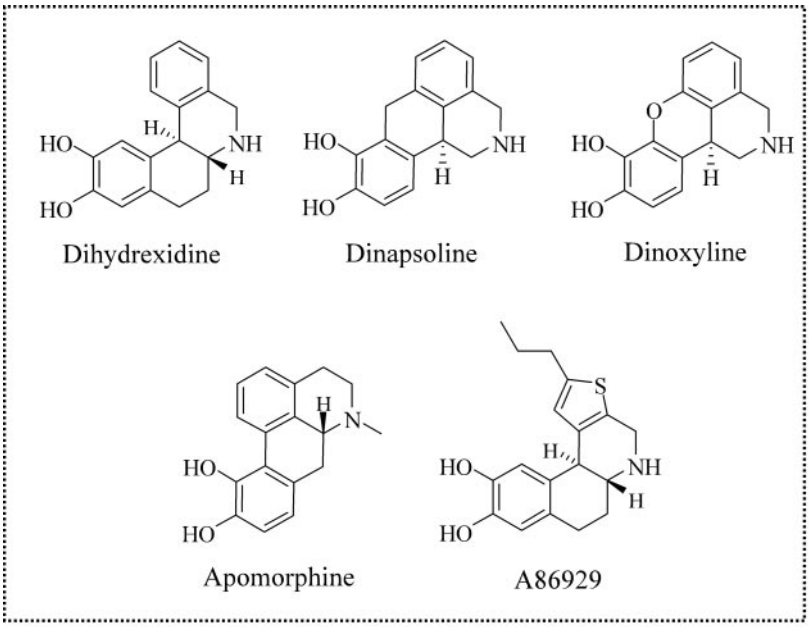
ABBREVIATIONS: GPCR, G protein-coupled receptors; HA, hemagglutinin; A68930, (1*R*,3*S*)-3-phenyl-1-aminomethyl-3,4-dihydo-5,6-dihydroxy-1*H*-2-benzopyran; A77636, (1*R*,3*S*)-3-(1′adamantyl)-1-aminomethyl-3,4-dihydo-5,6-dihydroxy-1*H*-2-benzopyran; A86929, (5*aR*,11*bS*)-4,5,5*a*,6,7,11*b*-hexahydro-2-propyl-3-thia-5-azacyclopent-1-ena[*c*]-phenanthrene-9,10-diol; DHX, *trans*-10,11-dihydroxy-5,6,6*a*,7,8,12*b*-hexahydrobenzo[*a*]phenanthridine; DNS, 8,9-dihydroxy-2,3,7,11*b*-tetrahydro-1*H*-naph[1,2,3-*de*]isoquinoline; DNX, 8,9-dihydro-1,2,3,11*b*-tetrahydrochromeno[4,3,2,-*de*]isoquinoline; GRK, G protein-coupled receptor kinase; IBMX, isobutylmethylxanthine; $K_{0.5}$, concentration-corrected IC_{50} (apparent affinity constant) when $n_H \neq 1.0$; HEK, human embryonic kidney; PBS, phosphate-buffered saline; ANOVA, analysis of variance; SCH23390, 7-chloro-8-hydroxy-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine; SKF38393, 7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine; SKF77434, (±)-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine; SKF81297, *R*(+)-6-chloro-7,8,-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine; SKF82957, *R*(+)-6-chloro-7,8,-dihydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine; SKF82958, 6-chloro-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine; SKF83959, 3-methyl-6-chloro-7,8-hydroxy-1-[3-methylphenyl]-2,3,4,5-tetrahydro-]*H*-3-benzazepine.

nylate cyclase are effective anti-Parkinson agents (Mailman et al., 2001), and show promise in the treatment of a number of other neuropsychiatric disorders. However, the effects of these compounds on the regulation of other endpoints of D_1 receptor activation are not well understood.

Internalization in response to dopamine is an important early regulatory mechanism that regulates D_1 receptor responsiveness. This process has been well studied for dopamine in model cell lines and is probably mediated by the GRK/arrestin pathway (Tiberi et al., 1996; Zhang et al., 1999; Kim et al., 2004). The D_1 receptor is recycled rapidly back to the cell surface after internalization (Vickery and von Zastrow, 1999; Vargas and von Zastrow, 2004). Thus, internalization in response to the endogenous agonist is a mechanism by which the receptor can be rapidly desensitized/resensitized in vitro. Internalization in vivo has also been observed in striatal neurons under hyperdopaminergic conditions in both rats (Dumartin et al., 1998) and humans (Muriel et al., 1999), as well as after administration to rats of the partial agonist SKF82958 (Dumartin et al., 1998). Thus, D_1 receptor internalization is likely to be of important physiological relevance, yet this endpoint remains largely unaddressed for D_1 agonists.

In C-6 glioma cells stably transfected with the macaque D_1 receptor, we have observed that some D_1 agonists from different structural classes were able to mediate both desensitization and down-regulation of D_1 receptors independent of the intrinsic activity of these compounds at adenylate cyclase (Lewis et al., 1998). The disparate effects on down-regulation led to the hypothesis tested herein that structurally dissimilar D_1 agonists differentially regulate two early responses of D_1 receptor activation: stimulation of adenylate cyclase and receptor internalization. There is precedence for this hypothesis with other GPCR systems (Whistler et al., 1999) and some suggestions of this possibility for the D_1 receptor (Lewis et al., 1998; Vargas and von Zastrow, 2004). The ability of a ligand to cause different degrees of activation of signaling pathways initiated by a single receptor isoform has been given various names, including agonist trafficking (Kenakin, 1995) and functional selectivity (Mailman et al., 1998; Mailman and Gay, 2004).

The potential of D_1 agonists as therapeutic agents in a variety of central nervous system disorders ranging from Parkinson's disease (Taylor et al., 1991; Mailman and Nichols, 1998) to cognitive deficits (Arnsten et al., 1994; Goldman-Rakic et al., 2004) has led to the design and synthesis of structurally diverse D_1 receptor agonists with variable degrees of ability to activate adenylate cyclase. For the purposes of this work, we have classified these agonists structurally into three major families. The first group of agonists is structurally rigid and includes apomorphine, dihydrexidine, A86929, dinapsoline, and dinoxyline (Fig. 1). These



Ligand	R1	R2	R3
SKF38393	H	H	H
SKF77434	H	CH_2CHCH_2	H
SKF81297	Cl	H	H
SKF82958	Cl	CH_2CHCH_2	H
SKF82957	Cl	CH_3	H
SKF83959	Cl	CH_3	CH_3

Fig. 1. Dopamine and D_1 agonists used in this study. The rigid agonists (top), the benzazepines (bottom), and the isochromans (right) are grouped in boxes (broken lines).

compounds have fused ring systems, relatively restricted conformational mobility, and, with the exception of A86929, no rotatable bonds (Mottola et al., 1996). The second class is the "benzazepines", from which SKF38393, SKF77434, SKF82958, SKF83959, SKF82957, and SKF81297 were selected (Fig. 1). All of the benzazepines have similar stereochemistry and a necessary phenyl substituent that is conformationally mobile (Charifson et al., 1989; Mottola et al., 1996). The final class of agonists is the isochromans (Fig. 1), from which A68930 and A77636 were developed (DeNinno et al., 1990, 1991). All three classes of D_1 agonists have docking and activation modes that are relatively similar (Mottola et al., 1996).

This study tested the hypothesis that the ability of D_1 agonists to show functional selectivity in terms of either activating adenylate cyclase or causing agonist-induced internalization is related to the structural class of the agonist. In addition to testing this hypothesis, this study constitutes the most extensive characterization reported to date of the internalization of the D_1 receptor in response to structurally diverse agonists.

Materials and Methods

Materials. Dihydrexidine, dinapsoline, and dinoxyline were synthesized according to procedures published previously (Brewster et al., 1990; Ghosh et al., 1996; Grubbs et al., 2004). The D_1-selective antagonist [^{3}H]SCH23390 was synthesized according to procedures published previously (Wyrick and Mailman, 1985). A68930 and A86929 were gifts from Abbott Laboratories (Chicago, IL), and the remaining D_1 agonists, dopamine, A77636, SKF83959, SKF82958, SKF38393, SKF77434, SKF82957, SKF81927, and apomorphine were purchased from Sigma/RBI (Natick, MA), which was also the source of other reagents and materials unless otherwise stated.

Construction and Maintenance of the HA-hD_1 HEK Stable Cell Line. The human dopamine D_1 receptor was amplified by polymerase chain reaction from a human cDNA library with a 5′ complementary forward primer containing an EcoRI site and a 3′ complementary reverse primer containing an XhoI site. The resulting product was cloned into pcDNA3.1 (Invitrogen, Carlsbad, CA). The QuikChange kit (Stratagene, La Jolla, CA) was used as directed in conjunction with a mutagenic forward primer to insert the nine-amino acid HA sequence YPYDVPDYA after Met^1. The resulting product was subcloned into the viral vector pLXSN (BD Biosciences Clontech, Palo Alto, CA). Twenty micrograms of the resulting HA-hD_1 construct was then transfected into the packaging cell line PA317, and the secreted virus was used to infect wild-type HEK cells as described previously (Comstock et al., 1997). Geneticin (Invitrogen) at 0.6 mg/ml was used for selection. HA-hD_1 HEK cells were maintained in high-glucose Dulbecco's modified Eagle's medium, 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.6 mg/ml geneticin at 37°C and 5% CO_2. Assay plates were coated with 4 μg/ml human fibronectin (Enzyme Research Laboratories Inc., South Bend, IN) at 37°C for 1 h or overnight followed by the addition of polylysine to 0.2 mg/ml for an additional 30 min to prevent cell loss. The coating medium was then aspirated, and cells were immediately plated at a density of 500 to 1000 cells/mm^2. Saturation binding with the D_1-selective antagonist [^{3}H] SCH23390 in membrane homogenates indicated that the assay expression level of HA-hD_1 in this cell line is approximately 4 ± 1 pmol/mg protein with a K_D of 2.4 ± 0.8 nM.

Competition Binding Experiments. The affinity of the HA-hD_1 receptor for various agonists was determined by competition binding assays. Membrane homogenates of HA-hD_1 HEK cells were prepared as described previously (Lewis et al., 1998). Duplicate wells were incubated with increasing concentrations of agonist and with 1 nM [^{3}H]SCH23390 in 50 mM HEPES, 4 mM $MgCl_2$, and 0.01% ascorbic acid, pH 7.4. Yohimbine and propranolol (50 nM) were included to block α- and β-adrenergic receptors. Nonspecific binding was determined by parallel incubations with 1 μM SCH23390 (Machida et al., 1992). Reactions were incubated for 15 min at 37°C. Binding reactions were terminated by rapid filtration onto 96-well filter plates (PerkinElmer Life and Analytical Sciences, Boston, MA) and washed with several volumes of ice-cold incubation buffer in the cell harvester. Microscint 20 (PerkinElmer Life and Analytical Sciences) scintillation fluid was then added to each well, and plates were counted for tritium on a TopCount (PerkinElmer Life and Analytical Sciences). Data were fit to a one-site competition model using Prism versions 3 and 4 (GraphPad Software Inc., San Diego, CA), and $K_{0.5}$ values for each agonist were calculated using the Cheng-Prusoff equation (Cheng and Prusoff, 1973).

Adenylate Cyclase Experiments. Adenylate cyclase activation by agonists was measured in whole HA-hD_1 HEK cells rather than membranes to make the parallel with the internalization as physiologically unconfounded as possible. Twenty-four–well plates of confluent HA-hD_1 HEK cells were equilibrated in L15 medium with 20 mM HEPES and 0.01% ascorbic acid, pH 7.4, for 1 h in a circulating water bath at 30°C. Plates were rapidly cooled on ice, and an equal volume of ice-cold medium [containing agonist (at twice the desired final concentration of 10^{-12} to 10^{-4} M), along with IBMX (1 mM), propranolol, and yohimbine (100 nM each to block endogenous adrenergic receptors)] was then added to triplicate wells. IBMX was used to inhibit phosphodiesterase on the basis of prior work from our laboratory on its validity in this assay (Schulz and Mailman, 1984). Duplicate wells that contained the highest concentration of D_1 agonist also had SCH23390 added to a final concentration of 50 μM. Plates were returned to the water bath for exactly 15 min (for which time cAMP response remains linear; data not shown). At the end of the incubation period, wells were washed once on ice with cold PBS, and the reactions were quenched with 0.1 N HCl. The cAMP content in an aliquot from each well was measured in duplicate by a competition radioimmunoassay with ^{125}I-labeled cAMP-sucTME as described previously (Harper and Brooker, 1975). Data were expressed relative to the percentage of maximal dopamine-mediated production of cAMP and were then fit to a sigmoidal dose-response curve; and EC_{50} values were calculated using Prism software (versions 3 and 4; GraphPad Software Inc.).

Internalization Experiments. Twenty-four–well plates of 1-day postconfluent HA-hD_1 HEK cells were adapted to treatment medium (L15 medium, 20 mM HEPES, and 0.01% ascorbic acid, pH 7.4) for 1 h. One plate per treatment time point was used. Each plate contained control wells for no drug (n = 6), drug plus antagonist (n = 2 per drug), and drug alone (n = 4 per drug). Dopamine or agonist was added to a final concentration of 10 μM. For wells containing drug plus antagonist, the final antagonist concentration (SCH23390 or butaclamol) was 50 μM. Propranolol and yohimbine (50 nM) again were included in all wells to block endogenous adrenergic receptors. Plates were incubated for 0 to 120 min in a 37°C water bath, rapidly cooled on ice, and fixed in 4% paraformaldehyde buffered with 0.1 N sodium phosphate before analysis for cell-surface receptors by radioimmunoassay. Control studies also were performed in which the HA-hD_1 receptors were prelabeled with the HA.11 primary antibody before agonist treatment and fixation. This experiment yielded results similar to those of the studies above, and the data are not shown.

Radioimmunoassay for Cell-Surface HA-hD_1. A protocol derived from that of Brinson and Harden (2001) was followed. All wells were blocked for 30 min in high-glucose Dulbecco's modified Eagle's medium containing 50 mM HEPES, 10% heat-inactivated fetal bovine serum, and 0.01% sodium azide at 37°C and 5% CO_2 in the cell-culture incubator. Primary antibody to HA.11 (mouse raw acytes; Covance, Princeton, NJ) then was added at a 1000-fold dilution to all wells except for two "no drug" wells that allowed estimation of background binding of radioactive, secondary antibody. Plates were returned to the incubator for 2 h. Wells were rinsed twice with

PBS, and a radioactive secondary antibody (^{125}I-labeled rabbit anti-mouse; PerkinElmer) was added at a 500-fold dilution to all wells, after which plates were returned to the incubator for an additional 2 h. Cells then were washed twice with PBS, solubilized overnight in 1 N NaOH, transferred to borosilicate test tubes, and radioactivity was quantified on an LKB 1209 RackBeta (PerkinElmer Wallac, Wellesley, MA). Data were expressed as a percentage of untreated controls.

Data Analyses. Affinity data were fit first to a sigmoidal model of variable slope. $K_{0.5}$ values were calculated from the IC_{50} values using the bimolecular competitive model from Cheng and Prusoff (1973). The data presented represent the means (± S.E.M.) of three to five independent experiments. The data then were resolved assuming a two-site model to yield $K_{D(high)}$ and $K_{D(low)}$ values and a percentage of sites in each state. The data for adenylate cyclase also were fit to a sigmoidal dose-response curve (variable slope). Intrinsic activity data for adenylate cyclase are expressed as the percentage of cAMP produced at the highest concentration of agonist used relative to dopamine. Intrinsic activity data for internalization are expressed as the percentage of remaining cell-surface receptors relative to dopamine-treated cells 2 h after agonist treatment. All values are the means and standard errors of two to five independent experiments. All of the analyses of these dose-response data were performed using Prism version 3 or 4 (GraphPad Software Inc.).

Differences between more than two groups were subjected to analysis of variance (ANOVA). Significant ANOVA results were followed by a post hoc Tukey test. Systat software (version 6.0.1; Systat Software Inc., Point Richmond, CA) was used for all statistical analysis.

Results

Affinity of Structurally Distinct Ligands for the HA-hD$_1$ Receptor. The affinity of the D_1 agonists was deter-

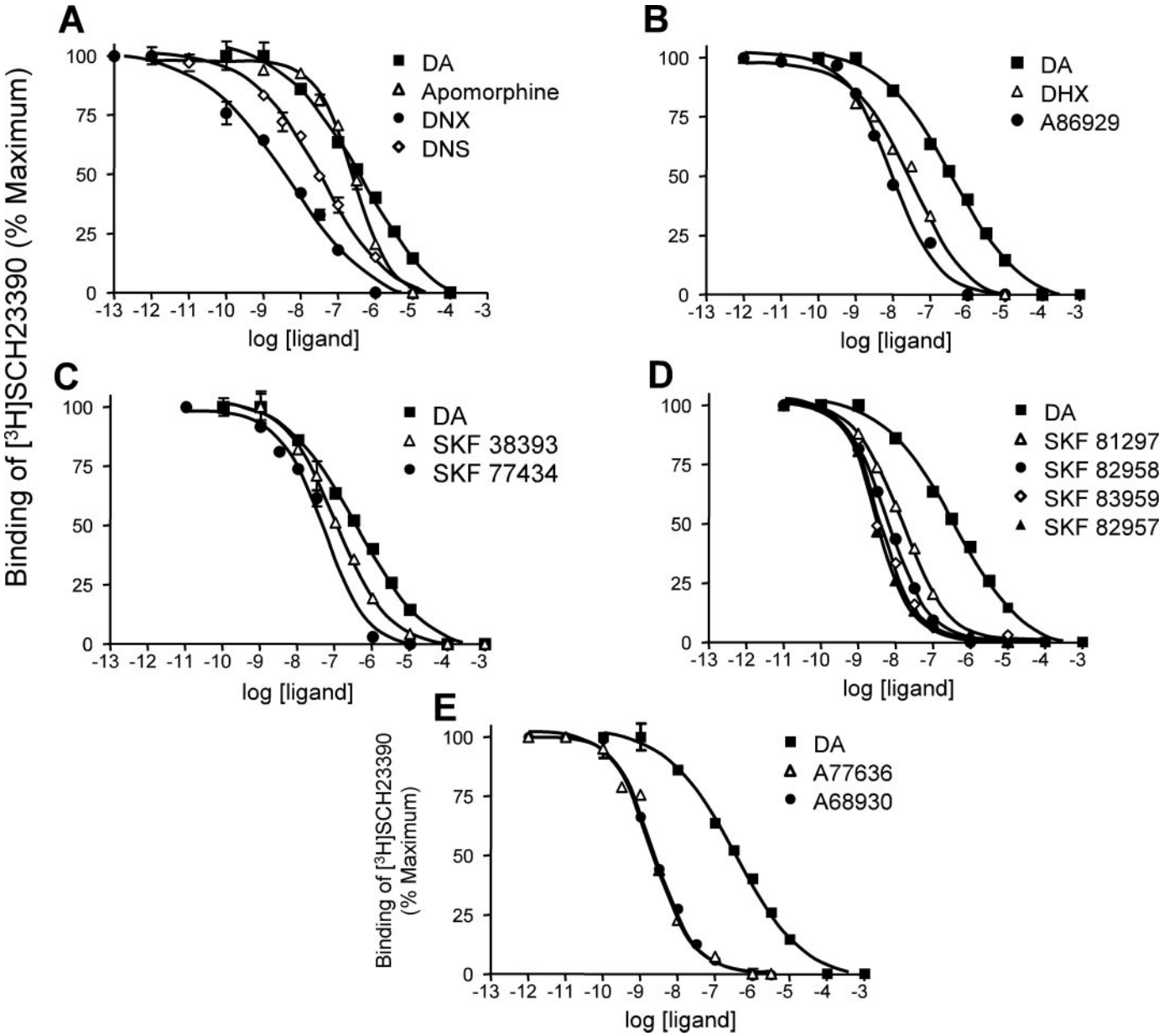


Fig. 2. Affinities of ligands from different structural classes for the HA-hD$_1$ receptor. Membrane homogenates of HA-hD$_1$ HEK cells were incubated with the D_1-selective antagonist [^{3}H]SCH23390 and increasing concentration of agonist. Data were fit to a one-site competition curve. Competition curves are from one experiment that is representative of two independent experiments. A and B, rigid agonists. C and D, benzazepine agonists. E, isochroman agonists. $K_{0.5}$ values are listed in Table 1.

mined for the HA-hD_1 receptor by competition binding with [^{3}H]SCH23390 (Fig. 2 and Table 1). This cell line had a receptor density of 4 ± 1 pmol receptor/mg membrane protein and a K_D value for [^{3}H]SCH23390 of 2.4 ± 0.8 nM (data not shown). Dopamine had an affinity of ~300 nM, and apomorphine and the benzazepine SKF38393 were also of relatively low affinity. The other rigid agonists had higher affinity for the HA-hD_1 receptor (8–70 nM), as did the remaining benzazepines (SKF83959, 3 nM; SKF77434, ~50 nM). The isochroman agonists A77636 and A68930 both had high affinity (~2–4 nM). The data from ligands with shallow Hill slopes (<0.8) also were resolved using a two-site analysis. Several correlations were tested, but significant relationships were found neither between the affinity of the high-affinity site and intrinsic activity in the functional assays nor between the spread of the high and low-affinity sites and intrinsic activity.

Adenylate Cyclase Functional Potency and Intrinsic Activity. The ability of these agonists to activate adenylate cyclase through the HA-hD_1 receptor was then examined (Table 1 and Fig. 3). Full dose-response curves for adenylate cyclase activation were obtained for all agonists. Dopamine exhibited very high functional potency in this cell line (approximately 20 nM) that was inhibited by the D_1-selective antagonist SCH23390. Adenylate cyclase activation by the highest concentration of all agonists was also inhibited by SCH23390, confirming that this response was mediated by the HA-hD_1 receptor. All of the rigid agonists exhibited high intrinsic activity for adenylate cyclase activation (>80%), with potencies ranging from 2 nM (A77636) to 37 nM (apomorphine). As expected, there was no correlation between adenylate cyclase potency and intrinsic activity for agonists of this class. Intrinsic activity for the benzazepines ranged from ~40% (SKF83959) to approximately 85% (SKF82958), with potency from less than 1 nM (SKF82957) to 11 nM (SKF38393). Not surprisingly, there also was no correlation between functional potency and efficacy for the benzazepines. The two isochromans tested, A77636 and A68930, had high intrinsic activity (>90%) and high potency (low nanomolar range).

Internalization Intrinsic Activity. D_1 agonists from different structural classes were then screened for their ability to cause the HA-hD_1 receptor to internalize (Figs. 4 and 5). Internalization for all agonists that caused this response was blocked by SCH23390 (data not shown), indicating that this effect was a direct consequence of D_1 receptor binding. The rigid agonists varied in the degree and time course of receptor internalization. Dinapsoline and A86929 caused internalization of the receptor to an extent similar to that observed with dopamine. The internalization response, however, was slower than that of dopamine for A86929 and more rapid than that of dopamine for dinapsoline. The extent of internalization of the receptor in response to dinoxyline was significantly greater than that observed for dopamine ($p < 0.01$) and occurred on a faster time scale than that observed with any other agonist. No internalization of HA-hD_1 was observed in response to apomorphine. The benzazepine compounds also varied in receptor internalization efficacy. Neither SKF83959, SKF82957, SKF38393, nor SKF77434 caused internalization of HA-hD_1. Internalization in response to two benzazepine agonists, SKF82958 and SKF81297, was observed. In both cases, the efficacy was similar to that of dopamine ($p > 0.05$) and followed a similar time course. The isochromans A77636 and A68930 both caused internalization of the HA-hD_1 receptor to an extent significantly greater than that observed with dopamine ($p <$

TABLE 1
Summary of pharmacological properties of structurally dissimilar D_1 agonists
Intrinsic activity data are expressed relative to dopamine. See *Materials and Methods* for details of analysis.

Ligand	Affinity				Adenylate Cyclase		Internalization Intrinsic Activity
	$K_{0.5}$	n_H	$K_{D(High)}$ (nM) R_{High} (%)	$K_{D(Low)}$ (nM) R_{Low} (%)	EC_{50}	Intrinsic Activity	
	nM				*nM*		
Dopamine	300 ± 120	−0.45 ± 0.04	19 ± 0.30 46 ± 3.0	2100 ± 38 44 ± 3.0	23 ± 18		
Apomorphine	240 ± 76	−0.85 ± 0.04	0.49 ± 0.03 7.6 ± 2.0	240 ± 2.0 92 ± 2.0	37 ± 3	86 ± 4	0.0
Dinapsoline	48 ± 5.0	−0.46 ± 0.05	1.4 ± 0.03 43 ± 6.0	140 ± 3.6 57 ± 6.0	4.0 ± 3.0	100 ± 18	106 ± 3
Dinoxyline	16 ± 9.3	−0.40 ± 0.05	0.12 ± 0.01 44 ± 4.0	30 ± 0.46 56 ± 4.0	4.0 ± 3.0	102 ± 14	118 ± 2
Dihydrexidine	71 ± 8.5	−0.63 ± 0.09	0.06 ± 0.01 21 ± 3.0	25 ± 0.20 79 ± 3.0	15 ± 0.5	96 ± 8	110 ± 5
A86929	7.8 ± 1.5	−0.68 ± 0.06	2.5 ± 0.03 68 ± 7.0	110 ± 4.2 32 ± 7.0	2.3 ± 0.5	91 ± 15	108 ± 3
SKF38393	240 ± 140	−0.63 ± 0.05	14 ± 0.39 48 ± 11	370 ± 11 52 ± 11	13 ± 1.0	67 ± 1	0.0
SKF77434	52 ± 2	−0.75 ± 0.07	0.79 ± 0.04 18 ± 6.0	45 ± 0.63 82 ± 6.0	11 ± 2.0	46 ± 12	0.0
SKF83959	2.7 ± 0.7	−0.94 ± 0.10	N.A.	N.A.	5.6 ± 0.6	42 ± 3	0.0
SKF82957	2.5 ± 0.3	−1.00 ± 0.09	N.A.	N.A.	0.8 ± 0.6	55 ± 9	0.0
SKF81297	10 ± 1	−0.654 ± 0.05	1.8 ± 0.12 42 ± 8.0	36 ± 0.59 58 ± 8.0	2.6 ± 1.2	89 ± 11	105 ± 3
SKF82958	6.5 ± 0.2	−0.73 ± 0.05	1.2 ± 0.03 46 ± 14	14 ± 0.31 54 ± 14	3.0 ± 2.6	86 ± 12	105 ± 3
A77636	3.8 ± 1.3	−1.01 ± 0.07	N.A.	N.A.	5.3 ± 2.0	99 ± 7	118 ± 2
A68930	2.4 ± 0.2	−0.78 ± 0.05	0.90 ± 0.01 73 ± 0.07	18 ± 0.49 27 ± 0.02	0.3 ± 0.1	97 ± 3	124 ± 2

N.A., not applicable (n_H = 1).

0.01). These agonists, however, did follow an internalization time course similar to that of dopamine.

Discussion

This study provided the broadest comparison to date of the molecular pharmacology of structurally diverse dopamine D_1 agonists. All of the structurally rigid compounds and the isochromans (Fig. 1) had high intrinsic activity in stimulating adenylate cyclase. Not unexpectedly, there was no correlation between agonist intrinsic activity and adenylate cyclase potency, with EC_{50} values covering more than 2 orders of magnitude. Likewise, there was also no apparent correlation between functional potency and efficacy (versus adenylate cyclase) for the benzazepine agonists. Most of the compounds in this class had low or moderate efficacy (e.g., SKF83959 or SKF38393). The exception, SKF82958, was nearly a full agonist, as reported previously (Watts et al., 1995). This dissociation between agonist functional potency and intrinsic activity (i.e., for adenylate cyclase activation) for D_1 agonists is consistent with and extends results published previously (Lewis et al., 1998).

An assumption often made taken from classic pharmacological dogma is that strong agonists almost invariably have low affinity. We believe this notion is at odds with the concept of "functional selectivity", as can be seen from the current data. Dinapsoline, dihydrexidine, dinoxyline, A86929, A68930, and A77636 are all full agonists, equal in intrinsic activity to dopamine in all systems studied to date, even in

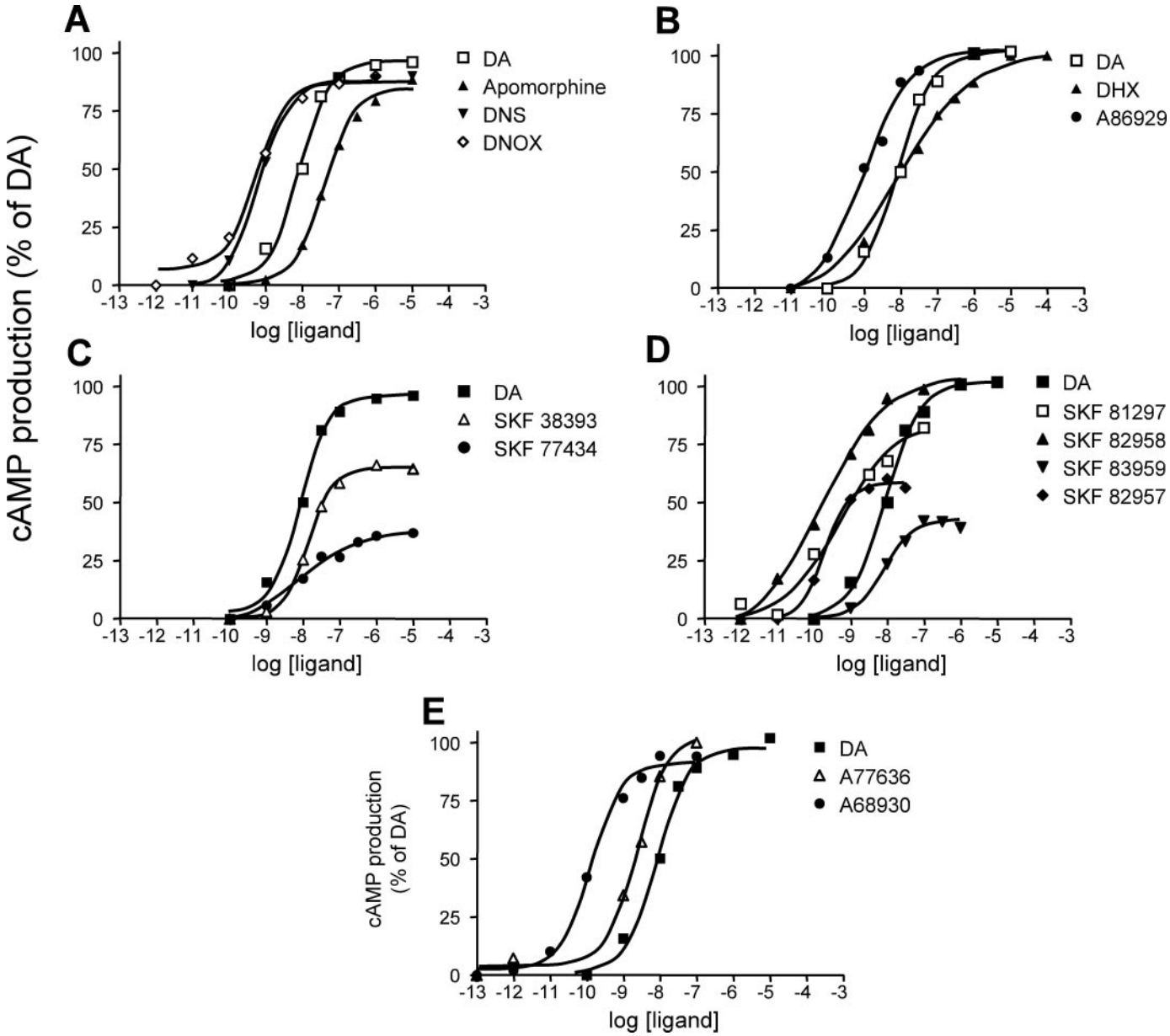


Fig. 3. Functional effects of agonists at adenylate cyclase at the HA-hD_1 receptor in whole HEK cells. HA-hD1 HEK cells were incubated with increasing concentrations of agonist in the presence of 500 μM IBMX, a phosphodiesterase inhibitor, and 50 nM (*S*)-propranolol and yohimbine (to antagonize endogenous adrenergic receptors). Data were fit to a variable slope sigmoidal dose-response curve. Curves are from one experiment that is representative of two or three independent experiments. The S.E.M. values in this study were less than 5% of the means. A and B, rigid agonists. C and D, benzazepine agonists. E, isochroman agonists. EC_{50} values are listed in Table 1.

those with reduced receptor reserve (Watts et al., 1995). Because these six ligands and dopamine have identical V_{max} values, the EC_{50} values directly reflect the EC_{50}/V_{max} ratios. Yet these six compounds have affinities and potencies that vary over a more than 30-fold range (even greater if dopamine is included). Examination of the data for partial agonists from the phenylbenzazepine family shows that they also cover approximately the same range of affinity and potency as the full agonists. Thus, whether full or partial agonist, all of these synthetic ligands are of relatively high affinity, contrary to what classic principles sometimes teach. As novel ligands are discovered for many receptors, more data are being published showing that functional activity is not correlated to ligand affinity. It would seem, therefore, that the parsimonious conclusion is that binding dynamics can result in a broad landscape of resulting receptor conformational changes that may (or may not) activate different signaling pathways in a differential fashion. Such data underscore why we believe that the acceptance of the concept of functional selectivity (or whatever name the field ultimately adopts) is long overdue.

Of greater interest is the relationship between agonist functional potency and the ability to cause receptor internalization. Some agonists were unable to cause internalization of the HA-hD_1 receptor, even when internalization was observed with other agonists of similar structural features. Thus, apomorphine, despite having relatively high intrinsic activity in stimulating cyclase, failed to cause receptor internalization, whereas all of the other rigid agonists (e.g., dinapsoline, A86929, and dinoxyline) elicited an internalization response of similar or greater magnitude than that observed with dopamine. The benzazepines SKF38393 and SKF77434, as well as SKF82957 and SKF83959, also failed to cause receptor internalization, whereas the other two members of this structural class (SKF82958 and SKF81297) had internalization efficacies similar to that of dopamine.

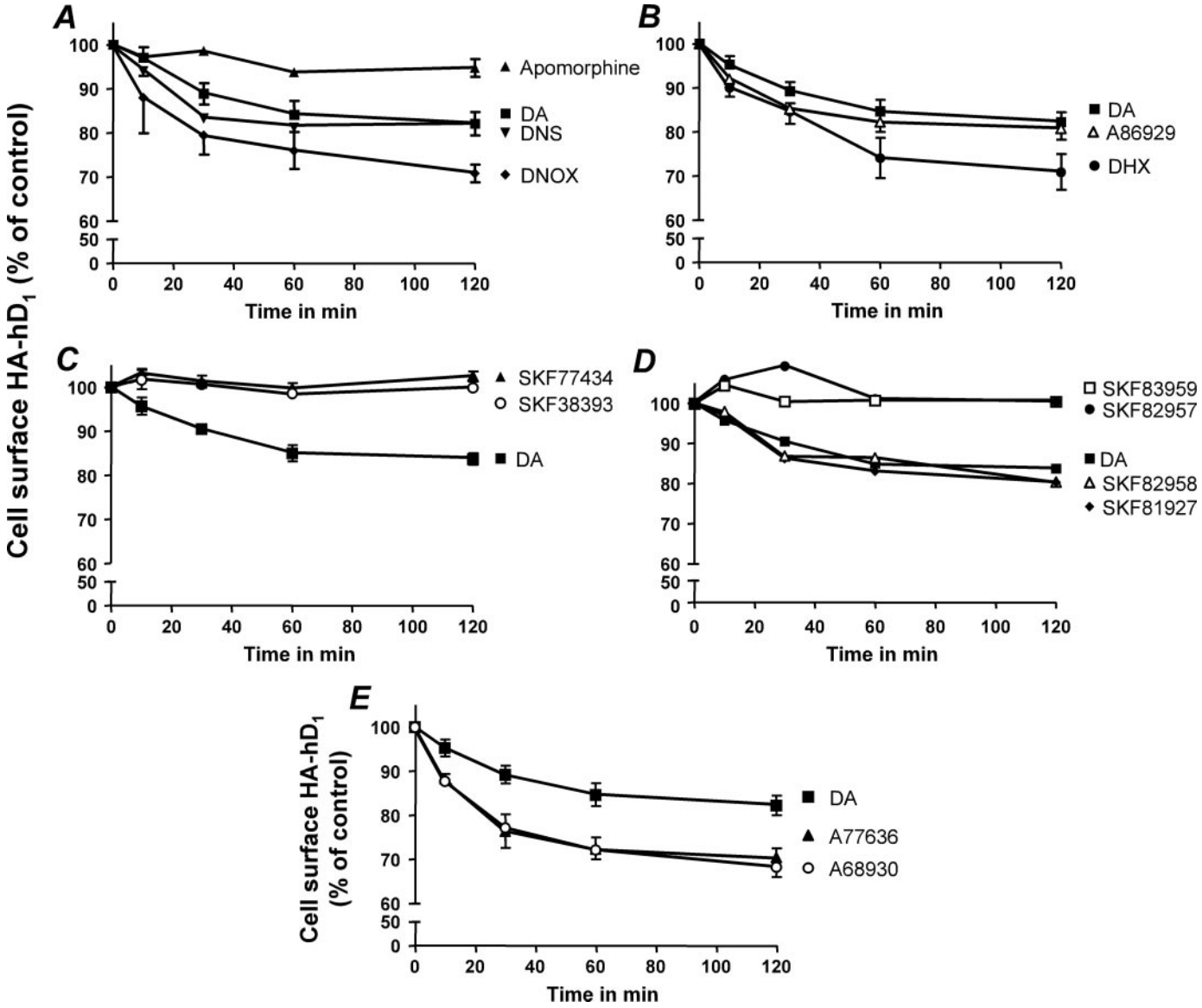


Fig. 4. Internalization time course of dopamine relative to other structurally dissimilar D_1 agonists. A and B, rigid agonists. C and D, benzazepine agonists. E, isochroman agonists. HA-hD1 HEK cells were treated with 10 μM agonist for 0 to 120 min. Cells were fixed, and remaining cell-surface HA.11 was determined by radioimmunoassay. Data were expressed as a percentage of vehicle-treated controls at all time points. Data are the means and standard errors of three to five independent experiments, except for SKF82957 ($n = 2$).

One of the surprising findings of this study was that internalization seemed to be almost a digital rather than a graded response (i.e., one either saw internalization to the same extent as dopamine, or one did not). This observation led to the hypothesis that the apparent inability of some of these agonists to cause D_1 receptor internalization was caused by the fact that the HA-hD_1 receptor could rapidly recycle back to the cell surface, even in the presence of agonist. Such an effect would result in no apparent internalization, because the cell-surface radioimmunoassay was performed on HA-hD_1 fixed after agonist exposure. We addressed this possibility by performing experiments in which the HA-hD_1 receptor was prelabeled with the HA.11 primary antibody before agonist treatment and fixation. However, this manipulation had no effect on the internalization efficacy of these agonists, thus ruling out this alternate hypothesis

Another concern was that the apparent high receptor reserve in this expression system (e.g., evidenced by the relatively high receptor density and by the high functional potency for stimulating adenylate cyclase) might be an interpretational confound. Yet as summarized in Table 1, the agonists used offered a broad range of intrinsic activities versus adenylate cyclase, indicating that despite the postulated high receptor reserve, it was still possible to discriminate full and partial agonists, at least at adenylate cyclase. In addition, all of the agonists tested in this assay system that caused D_1 receptor internalization exhibited an internalization efficacy that was equivalent to or greater than that of dopamine. These results indicate that the postulated high receptor reserve did not hinder our ability to detect agonist-specific differences in receptor internalization. Furthermore, the observed dissociation between the adenylate cyclase activity and internalization efficacy of an agonist also speak against a role for cAMP and protein kinase A in mediating D_1 receptor internalization.

In particular, apomorphine had intrinsic activity for stimulating adenylate cyclase that was identical with SKF81297 and SKF82958, yet these two benzazepines caused full internalization, whereas apomorphine caused none. It should be noted that the high D_1 intrinsic activity we report for apomorphine in vitro (in addition to its well-known D_2 agonism) is consistent with the fact that it causes profound anti-Parkinson effects in vivo, a characteristic of full but not partial D_1 agonists (Taylor et al., 1991; Mailman et al., 2001). Another interesting finding in this study was the identification of three agonists from two different structural classes that were more effective than dopamine in causing internalization of the HA-hD_1 receptor. The rigid agonist dinoxyline, as well as both of the isochromans, A86929 and A68930, caused greater internalization than did dopamine for reasons that are not obvious.

One of the initial hypotheses of this study was that dopamine D_1 receptor agonists from the same structural class might have similar efficacy and/or time courses for causing D_1 receptor internalization. One of the rationales underlying this hypothesis was as follows. Although all of these agonists dock to a very similar pharmacophore (Mottola et al., 1996), we expected that subtle differences among the structural families would cause differences in functional selectivity. These data, however, clearly show that D_1 receptor internalization is not directly related to agonist structure or affinity for the D_1 receptor. Although there was no direct relationship to either intrinsic activity or functional potency (both assessed by activation of adenylate cyclase), our data suggest that together, these factors may somehow be related to whether or not a ligand can cause internalization of the D_1 receptor.

Although these interesting results clearly demonstrate functional selectivity at the dopamine D_1 receptor, they raise questions about the underlying mechanisms of differential activation of adenylate cyclase versus receptor internalization. G protein activation is the first step at which such discrimination could occur. Only agonists with both relatively high affinity and potency at adenylate cyclase activation were able to mediate an internalization response. In particular, all "internalizing" agonists displayed adenylate cyclase functional potency of at least 15 nM and intrinsic activity of at least 90%. Internalization was not observed for agonists with high potency but low intrinsic activity (SKF83959, 5.6 nM and 42% intrinsic activity) or low potency

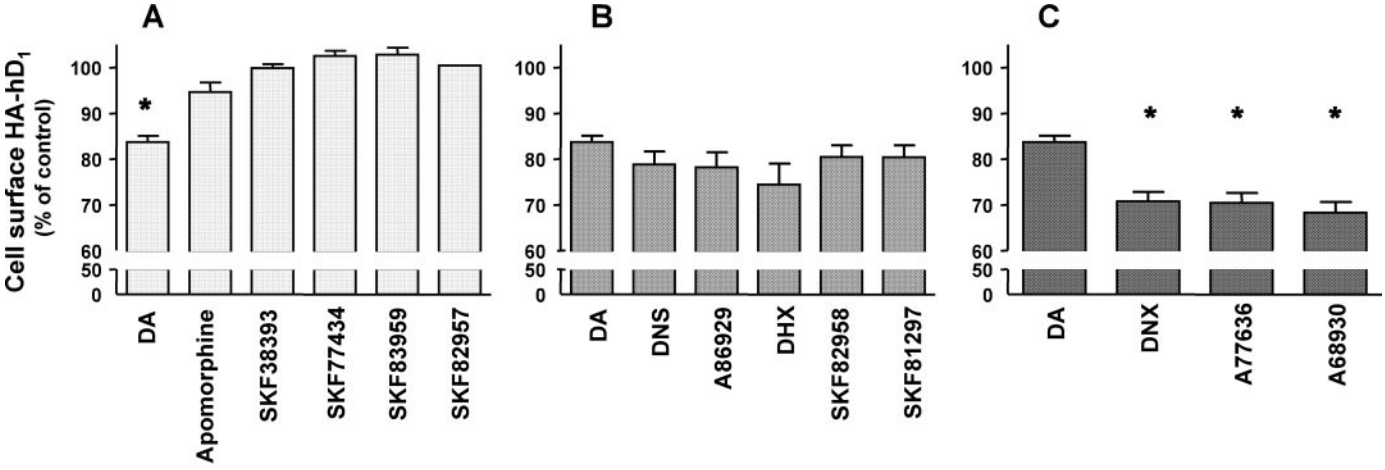


Fig. 5. Cell-surface receptors remaining 2 h after agonist treatment. Data were expressed as a percentage of vehicle-treated controls at all time points. Data are the means and standard errors of three to five independent experiments, with the exception of SKF82957 ($n = 2$), and are shown relative to dopamine. Data were compared using a one-way ANOVA with a post hoc Tukey test. A, noninternalizing agonists. Internalization of HA-hD_1 is significant for dopamine compared with all other agonists ($\star$, $p < 0.001$). B, agonists that cause internalization similar to that observed with dopamine ($p > 0.05$). C, agonists of higher internalization efficacy than dopamine. Internalization of all compounds is significantly greater than that observed with dopamine ($p < 0.01$).

but high intrinsic activity (apomorphine, 37 nM and 86% intrinsic activity). On the surface, these findings suggest that differences in the ability of the agonist-receptor complex to couple efficiently to a stimulatory G protein may be an important mechanism underlying functional discrimination by D_1 agonists. Nevertheless, the lack of a graded response for the partial agonists (i.e., at adenylate cyclase) suggests that the underlying mechanism is likely to be more complex.

Indeed, functional discrimination may be independent of G protein activation. The δ and κ opioid receptors, for example, activate inhibitory G proteins with similar potency and efficacy in response to etorphine in HEK cells, but the κ receptor fails to cause an internalization response, a fact attributed to sequence differences in the receptor C termini that regulate internalization (Chu et al., 1997). On the other hand, the ability of the agonist-receptor complex to regulate both the extent of G protein activation and downstream events may prove important in regulating differential activation of adenylate cyclase and internalization. Dopamine has been observed previously to cause D_1 receptor internalization in this cell line through a dynamin-dependent mechanism that implicates the involvement of the GRK/arrestin/clathrin pathway (Vickery and von Zastrow, 1999). Such an operative mechanism would be consistent with a requirement for G_s activation, because GRKs phosphorylate only the activated form of GPCRs (Ferguson et al., 1996). In addition, this mechanism requires that cellular GRKs be able to bind to and phosphorylate the agonist-occupied form of the receptor and that cellular arrestins also be able to bind. The promiscuity of GRK phosphorylation and arrestin binding would be consistent with one cellular mechanism by which internalization of the receptor could be regulated in response to a number of structurally diverse agonists. This same mechanism also offers a means to explain why small perturbations in agonist structure, as observed with the benzazepines, could inhibit internalization, even when activation of a stimulatory G protein is present. These possibilities are worthy of future study, noting of course that in many systems G_{OLF}, rather than G_s, may the important mediator of D_1 activity (Herve et al., 1993; Corvol et al., 2001).

In summary, these experiments screened a number of agonists from three different structural families and identified D_1 agonists that activate adenylate cyclase but do not cause receptor internalization. These effects were independent of agonist structural class and agonist affinity and indicated a role for efficient and potent mobilization of a stimulatory G protein. The data also suggest that a similar internalization mechanism underlies these results (possibly one mediated by cellular GRKs/arrestin) but do not rule out the possibility that these effects may be caused by differential G protein coupling. This study also constitutes the most extensive characterization reported to date of D_1 receptor internalization in response to structurally diverse agonists.

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