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Pivotal Role for Aspartate-80 in the Regulation of Dopamine D2 Receptor Affinity for Drugs and Inhibition of Adenylyl Cyclase

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SUMMARY

An aspartate residue corresponding to aspartate-80 of dopamine D2 receptors is strictly conserved among receptors that couple to guanine nucleotide-binding proteins. Mutation of this residue alters the function of several classes of neurotransmitter receptors. Dopamine D2 receptors couple to the guanine nucleotide-binding protein G_i to inhibit adenylyl cyclase (ATP-pyrophosphate-lyase, cyclizing; EC 4.6.1.1). Like other G_i-coupled receptors, the binding of agonists and some antagonists to D2 receptors is sensitive to pH and sodium. In the present report, we demonstrate that substitution of an alanine or glutamate residue for aspartate-80 severely impairs inhibition of adenylyl cyclase by D2 receptors and also abolishes or decreases the regulation of the affinity of D2 receptors for agonists and substituted benzamide antagonists by sodium and pH. Our data support the hypothesis that the conformation of D2 receptors is maintained by interactions of monovalent cations with aspartate-80. The regulation of D2 receptors by this interaction has important consequences for the affinity of D2 receptors for ligands and for signal transduction by D2 receptors.

DA D2 receptors couple to G_i to attenuate the activity of adenylyl cyclase (1, 2). Among the characteristics of G_i-coupled receptors that are shared by D2 receptors is the ability to modulate several signaling pathways (3). In addition to inhibiting adenylyl cyclase activity, activation of D2 receptors has been reported to inhibit or stimulate hydrolysis of polyphosphoinositides (4, 5), increase K^+ conductance (6), inhibit Ca^{2+} channels (7), and decrease Na^+/H^+ exchange (8).

G_i-coupled receptors also have in common the property of sensitivity to sodium. Regulation of DA D2 receptors by sodium is reflected in several phenomena. First, sodium enhances inhibition of adenylyl cyclase activity by D2 receptors (2). Second, in receptor binding assays carried out using membrane preparations, sodium decreases the affinity of D2 receptors for agonists (9, 10). Third, sodium greatly increases the affinity of D2 receptors for substituted benzamide antagonists (11, 12). The affinity of DA D2 receptors for agonists and substituted benzamides is also altered by variations in $[H^+]$ between pH 6.8 and 7.5 or 8 (13). These effects of pH and Na^+ on DA D2 receptors may be related to the ability of D2 receptors to inhibit Na^+/H^+ exchange (8).

We have proposed that this modulation of the affinity of D2 receptors for ligands results from a Na^+- or H^+-dependent receptor isomerization. Thus, decreases in $[Na^+]$ or pH accelerate the dissociation of the substituted benzamide ligand [^{125}I] epidepride from D2 receptors, as would be expected for an allosteric interaction in which the conformation of the receptor depends on the concentrations of Na^+ and H^+ (12, 13). Furthermore, inactivation of D2 receptors by NEM is inhibited by high $[H^+]$ (pH 6.8) or $[Na^+]$, indicating that the accessibility of a target for alkylation by NEM is modified by a Na^+- or H^+-dependent conformational change (13–15).

For α_2-adrenergic receptors, increases in $[Na^+]$ or $[H^+]$ lower the affinity of the receptors for agonists by acting directly on the receptors (16). The site of action of sodium is intracellular, so that modulation of cytoplasmic $[Na^+]$ alters the binding of agonists to α_2-adrenergic receptors on intact cells (17). The binding of agonists to D2 receptors is regulated by sodium even after inactivation of G proteins by pertussis toxin (10) or alkylation (18), indicating that an interaction between G proteins and D2 receptors is probably not involved in the regulation of ligand binding by sodium. We now report that replace-

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ABBREVIATIONS: DA, dopamine; G_i, inhibitory guanine nucleotide-binding protein; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NPA, propylnorapomorphine; NEM, *N*-ethylmaleimide; NMDG, *N*-methyl-D-glucamine; G protein, guanine nucleotide-binding protein; PCR, polymerase chain reaction.

ment of Asp-80 in D2 receptors with an alanine residue results in the expression of a receptor that is insensitive to changes in [Na^+] and has reduced sensitivity to changes in [H^+]. Furthermore, the mutant receptor is no longer able to inhibit adenylyl cyclase activity, suggesting that signal transduction by D2 receptors involves maintaining or adopting a particular receptor conformation as a result of the action of H^+ or Na^+ at Asp-80.

Materials and Methods

Site-directed mutagenesis of D2 receptors. Trans-PCR, which is used to join DNA fragments that contain a region of overlap, was used to perform site-directed mutagenesis by employing primers that contain the desired base substitutions. To create the alanine substitution for Asp-80, we made the oligonucleotide 5′-CTTGCTGTGGCTGCTCTTCTGGTG-3′ and its reverse complement, which changes GAT (Asp) to GCT (Ala). To create the glutamate substitution for Asp-80, we made the oligonucleotide 5′-CTTGCTGTGGCTGAACTTCTGGTG-3′ and its reverse complement, altering GAT to GAA (Glu). Each mutation was carried out in two steps. In the first step, the fragments to be joined were amplified in separate reactions, in which the sense mutated primer was paired with a downstream unmutated antisense oligonucleotide primer and the antisense mutated primer was paired with an upstream wild-type sense primer. Both reactions used 1 ng of $D2_{415}$ cDNA as template. In the second step, PCR was performed using a small amount of each product from the first reactions, together with the outer primers. The PCR for both steps was done under standard conditions (94° for 1 min, 60° for 2 min, 72° for 3 min) for 20 cycles. The PCR product was extracted with phenol and chloroform, precipitated using ethanol, and digested to completion with *Xho*I (within the 5′ end of the PCR fragment) and *Bgl*II (within the 3′ end of the fragment), yielding a 1.3-kilobase fragment including virtually all of the coding region of $D2_{415}$. This fragment was gel purified and ligated to pRSV-$D2_{415}$, which had been cleaved with the same enzymes and gel purified away from its wild-type *Xho*I-*Bgl*II insert. Confirmation of the mutations was obtained by sequence analysis of the clones.

DNA-mediated expression of mutant and wild-type D2 receptors in C_6 glioma cells. C_6 glioma cells were maintained as described previously (19), except that the medium was supplemented with 3% calf serum and 2% fetal bovine serum. Transfection of C_6 cells was carried out by calcium phosphate precipitation (20). Exponentially growing cells were seeded in 10 ml of Dulbecco's modification of Eagle's medium/10-cm plate and incubated overnight. $D2_{415}$ and $D2_{444}$ were cloned into pRSV (21), modified by the addition of a polylinker containing several unique restriction enzyme sites. Plasmid DNA (15 μg), composed of pRSV-D2 and pRSVneo (7:2), was mixed with 0.5 ml of 0.25 M $CaCl_2$. An equal volume of 2× BES-buffered saline [50 mM *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid, 280 mM NaCl, 1.5 mM Na_2HPO_4] was added, and the mixture was incubated at room temperature for 20 min before being added by drops to the medium in the plate. The plate was incubated for 20–24 hr in an atmosphere of 3% CO_2/97% air, washed with phosphate-buffered saline, re-fed, and incubated in an atmosphere of 10% CO_2. On the second day after addition of DNA, the cells were split into 10 plates and incubated overnight before the beginning of selection with G418 (600 μg/ml; Sigma). Clonal G418-resistant cells were isolated, after approximately 2 weeks of G418 treatment, by trypsinization within a 10-μl cloning ring, transferred to 96-well plates, and expanded into duplicate 60-mm plates. Cell lines that were positive for D2 receptors, determined by binding of [^{125}I] epidepride, were expanded into 10-cm plates for further characterization.

Radioiodination of epidepride (NCQ 219). (*S*)-(−)-*N*-[(1-Ethyl-2-pyrrolidinyl)-methyl]-2,3-dimethoxy-5-(tri-*n*-butyltin)benzamide (20 μg/20 μl of absolute ethanol) was mixed with Na[^{125}I] (10 mCi/20 μl). The solution was acidified by addition of 10 μl of 0.4 N HCl. Chloramine-T (20 μg/10 μl of water) was added, and the reaction proceeded for 3 min. The reaction was stopped by addition of sodium metabisulfite (380 μg/20 μl of water) and was then alkalinized. After extraction into ether, the product was purified by high performance liquid chromatography, using a cyano-silica column (100 × 4.6 mm; Waters, Radial PAK 8NYCN4HP) and a mobile phase of 38% ethanol/62% 20 mM potassium phosphate, pH 6.8. Radiochemical yield was 80–90%.

Radioligand binding assays. Cells were lysed by replacement of the growth medium with ice-cold hypotonic buffer (1 mM Na^+-HEPES, pH 7.4, 2 mM EDTA). After swelling for 10–15 min, the cells were scraped from the plate and centrifuged at 24,000 × *g* for 20 min. The resulting crude membrane fraction was resuspended with a Brinkmann Polytron homogenizer at setting 6 for 10 sec, in 5 mM K^+-HEPES (pH 7.4), and stored at −70° for receptor binding experiments. Aliquots of the membrane preparation were added to assay tubes containing (final concentrations) 50 mM Tris·HCl, pH 7.4, with 0.9% NaCl (Tris-buffered saline), except where indicated, 0.025% ascorbic acid, 0.001% bovine serum albumin, [^{125}I]epidepride (2000 Ci/mmol) or [^{3}H]spiperone, and appropriate drugs. In experiments to determine the effect of pH and Na^+ on binding affinity, Tris-buffered saline was not used. Reagents were added to each assay in water or 5 mM K^+-HEPES before the addition of 50 mM K^+-HEPES (final), pH 6.6 or 7.5, and 100 mM NaCl or 100 mM NMDG-Cl (22). (+)-Butaclamol (2 μM) or spiperone (1 μM) was used to define nonspecific binding in assays of the binding of [^{3}H]spiperone or [^{125}I]epidepride, respectively. GTP (100 μM) was added to assays in experiments assessing the binding of agonists. Assays were carried out in duplicate, except where indicated. Incubations were initiated by the addition of tissue, carried out at 30° for 60 min, and stopped by the addition of 10 ml of ice-cold wash buffer (10 mM Tris, pH 7.4, 0.9% NaCl) to each assay. The samples were filtered through glass fiber filters (Schleicher & Schuell no. 30) and washed with an additional 10 ml of wash buffer. The radioactivity retained on the filter was counted using a Beckman LS 1701 scintillation counter or a γ-counter (LKB Clinigamma 1272).

Equilibrium binding assays. Saturation experiments were carried out in a volume of 5 ml ([^{3}H]spiperone) or 0.25 or 0.5 ml [^{125}I] epidepride). Data were analyzed by nonlinear regression using the program GraphPAD. Competition experiments were carried out in a volume of 1 or 2 ml, using [^{3}H]spiperone as the radioligand. IC_{50} values were determined by nonlinear regression analysis using GraphPAD. In all competition experiments, the concentration of [^{3}H]spiperone ranged from approximately 120 to 180 pM. K_I values were calculated from experimentally determined IC_{50} values, as described by Munson and Rodbard (23). Averages for K_I and K_D values are expressed as pK_I or pK_D ± standard error. The standard Gibbs free energy change (ΔG°) was calculated from the equation $\Delta G^\circ = RT\ln K_D$, where R is the gas constant and T is the temperature in degrees Kelvin. Protein was measured by the method of Peterson (24).

Treatment with NEM. Membrane aliquots were incubated with 3 mM NEM at pH 6.6 or 7.5 (20 mM K^+-HEPES), in the presence of 50 mM NaCl or 50 mM NMDG. After 30 min at 37°, the reaction was quenched by addition of an equal volume of 6 mM dithiothreitol to each preparation, and the density of binding sites was determined by saturation analysis of the binding of [^{125}I]epidepride in the presence of 100 mM HEPES-buffered saline (pH 7.3). An aliquot (100 μl) of the membrane mixture was added to each 0.5-ml assay, for a final concentration of 0.3 mM NEM and 1.2 mM dithiothreitol. In preliminary experiments, these concentrations were determined to have no detectable effect on the binding of [^{125}I]epidepride.

Cyclic AMP accumulation. The accumulation of cyclic AMP in intact cells was measured as described previously (19). Cells were seeded in six-well cluster dishes at a density of 18,000/cm^2. On day 3, the growth medium was replaced by 1.5 ml of HEPES-buffered L15 medium, and the cells were incubated in air at 37° for 2 hr. [^{3}H]Adenine (1 μCi/well) was added to the incubation medium 15 min before drug or vehicle. Incubation with drugs was carried out for 7 min and was terminated by two rinses with ice-cold phosphate-buffered saline. [^{3}H]ATP and [^{3}H]cAMP were extracted in 3% trichloroacetic acid and

separated using successive Dowex and alumina columns. Results are expressed as the percentage of [^{3}H]ATP converted to [^{3}H]cAMP.

Results

Binding of [^{125}I]epidepride to D2$_{415}$ and D2$_{444}$. DA D2 receptor cDNAs encoding the D2$_{415}$ (415-amino acid) and D2$_{444}$ (444-amino acid) variants of D2 receptors were cloned into pRSV (21) and expressed by stable transfection into C_6 glioma cells. The affinity of D2$_{415}$ and D2$_{444}$ for [^{125}I]epidepride and the sensitivity of the two forms to changes in pH and [Na^+] were determined in membranes prepared from the cells. As shown in Table 1, the dependence on pH and [Na^+] of affinity values for binding of [^{125}I]epidepride to D2$_{415}$ was indistinguishable from D2$_{444}$.

TABLE 1

Effect of Na^+ and H^+ on affinity of D2$_{415}$ and D2$_{444}$ receptors for [^{125}I]epidepride

Affinity values, expressed as pK_D (the negative logarithm of the K_D value, in M), are given for the binding of [^{125}I]epidepride to membranes prepared from C6 glioma cells stably expressing either D2$_{415}$ or D2$_{444}$. Values shown are the means ± standard errors from three or four independent experiments. Experiments in which pH was varied were carried out in the presence of 120 mM NaCl, and experiments in which [NaCl] was varied were carried out at pH 7.4.

Assay condition	Affinity, pK_D	
	D2$_{415}$	D2$_{444}$
pH 7.5	10.61 ± 0.09	10.42 ± 0.13
pH 7.0	10.22 ± 0.11	10.18 ± 0.09
pH 6.8	9.86 ± 0.09	9.88 ± 0.04
50 mM NaCl	10.23 ± 0.01	10.30 ± 0.01
1 mM NaCl	9.83 ± 0.04	9.82 ± 0.07
0 NaCl	9.57 ± 0.06	9.61 ± 0.06

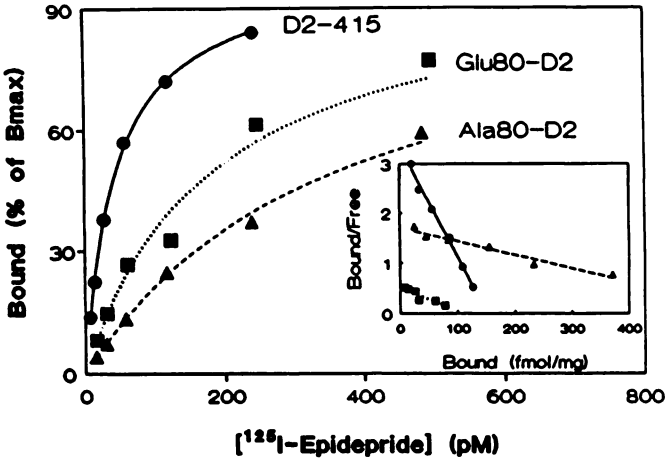

Fig. 1. Altered binding of [^{125}I]-epidepride to D2 receptors resulting from mutation of Asp-80. C_6 cell lines expressing either D2$_{415}$ or one of the mutant D2 receptors were isolated. Saturation isotherms of the binding of [^{125}I]-epidepride to membranes prepared from the cells are shown from a representative experiment. Assays were carried out at pH 7.4 in the presence of 120 mM NaCl. Results are plotted as radioligand bound, expressed as a percentage of B_{max} for each tissue, versus the corrected free concentration of [^{125}I]-epidepride (total radioligand added minus amount bound). In this experiment, affinity (K_D) and B_{max} values, respectively, for each tissue were 45 pM and 151 fmol/mg of protein (D2$_{415}$), 185 pM and 101 fmol/mg of protein (Glu-80-D2), and 367 pM and 626 fmol/mg of protein (Ala-80-D2). These data are shown here for graphic comparison of radioligand binding to wild-type and mutant D2 receptors but were not included in the mean pK_D and B_{max} values presented in the text. In those experiments, higher radioligand concentrations were used to characterize binding to Glu-80-D2 and Ala-80-D2, to obtain more accurate estimates for K_D and B_{max}. *Inset*, the same data, expressed as actual rather than normalized values, are transformed and plotted as radioligand specifically bound (fmol/mg of protein)/free radioligand (pM) versus radioligand specifically bound (fmol/mg of protein).

TABLE 2

Effect of H^+ and Na^+ on drug affinity for wild-type and mutant D2 receptors

The apparent affinity (K_i) of the indicated drugs was determined by inhibition of the binding of [^{3}H]spiperone. Affinity values for [^{3}H]spiperone are K_D values and were used to calculate K_i values for the other drugs. Data are expressed as pK_D or pK_i (the negative logarithm of the equilibrium dissociation constant, in M) ± standard error from three to five independent experiments. Experiments with the agonists DA and NPA were carried out in the presence of 100 μM GTP. Assays were carried out at the indicated pH in the presence of 100 mM NMDG-Cl (pH 6.6 and 7.5) or in the presence of 100 mM NaCl (pH 6.6/NaCl and pH 7.5/NaCl).

Drug	Affinity, pK_D or pK_i			
	pH 6.6/NaCl	pH 6.6	pH 7.5/NaCl	pH 7.5
[^{3}H]Spiperone				
D2$_{415}$	10.55 ± 0.01	10.41 ± 0.07	11.10 ± 0.05	10.97 ± 0.08
Ala-80-D2	10.70 ± 0.02	10.68 ± 0.08	11.08 ± 0.07	11.03 ± 0.10
Glu-80-D2	10.35 ± 0.07	10.43 ± 0.10	10.72 ± 0.12	10.78 ± 0.13
Epidepride				
D2$_{415}$	9.21 ± 0.12	8.51 ± 0.07	10.69 ± 0.05	9.67 ± 0.10
Ala-80-D2	8.47 ± 0.03	8.32 ± 0.03	9.33 ± 0.04	9.25 ± 0.03
Glu-80-D2	8.17 ± 0.11	7.97 ± 0.06	9.26 ± 0.12	9.00 ± 0.10
Sulpiride				
D2$_{415}$	6.31 ± 0.12	5.37 ± 0.03	7.96 ± 0.08	7.02 ± 0.02
Ala-80-D2	5.70 ± 0.04	5.65 ± 0.02	6.55 ± 0.05	6.45 ± 0.02
Glu-80-D2	5.45 ± 0.05	5.39 ± 0.03	6.54 ± 0.04	6.30 ± 0.02
NPA				
D2$_{415}$	7.73 ± 0.08	7.91 ± 0.07	8.37 ± 0.04	8.84 ± 0.13
Ala-80-D2	8.30 ± 0.14	8.31 ± 0.07	8.92 ± 0.10	8.78 ± 0.05
Glu-80-D2	7.33 ± 0.18	7.39 ± 0.40	7.52 ± 0.05	7.75 ± 0.04
DA				
D2$_{415}$	4.73 ± 0.02	4.72 ± 0.03	5.80 ± 0.06	6.09 ± 0.07
Ala-80-D2	5.08 ± 0.04	4.98 ± 0.04	5.78 ± 0.04	5.75 ± 0.05
Glu-80-D2	3.55 ± 0.03	3.42 ± 0.13	4.53 ± 0.04	4.22 ± 0.04

Binding of radioligands to wild-type and mutant D2 receptors. Two different point mutations were introduced into the D2$_{415}$ cDNA by the PCR, using a method termed trans-PCR (see Materials and Methods). Both mutations altered the DNA sequence encoding the amino acid residue Asp-80. The first mutation resulted in the replacement of Asp-80 with Ala-80 (Ala-80-D2) and the second in its replacement with Glu-80 (Glu-80-D2). Substitution of the aspartate residue with alanine was chosen because the small size of the side chain and the lack of reactive groups make alanine less likely to cause non-specific structural changes (25). The more conservative substitution of glutamate for aspartate might be expected to have less functional impact.

Cell lines that stably express mutant D2 receptors were created by transfection of C_6 glioma cells with pRSV-D2$_{415}$ into which a point mutation had been introduced. Saturation analysis of the binding of [^{125}I]epidepride to membranes prepared from the cells expressing mutant receptors and cells expressing D2$_{415}$ (Fig. 1) indicated that the density of binding sites (B_{max}) was 100 ± 2.5 fmol/mg of protein for D2$_{415}$ (four experiments), 53 ± 5 fmol/mg of protein for Glu-80-D2 (three experiments), and 496 ± 21 fmol/mg of protein for Ala-80-D2 (four experiments). The respective affinity (pK_D) values for D2$_{415}$, Glu-80-D2, and Ala-80-D2 were 10.36 ± 0.11, 9.73 ± 0.07, and 9.49 ± 0.14 pM. These experiments were carried out in the presence of 120 mM NaCl at pH 7.3. In contrast to the considerable decrease in D2 receptor affinity for [^{125}I]epidepride resulting from the substitution of an alanine residue for Asp-80, [^{3}H]spiperone bound with similar affinity values to D2$_{415}$, Glu-80-D2, and

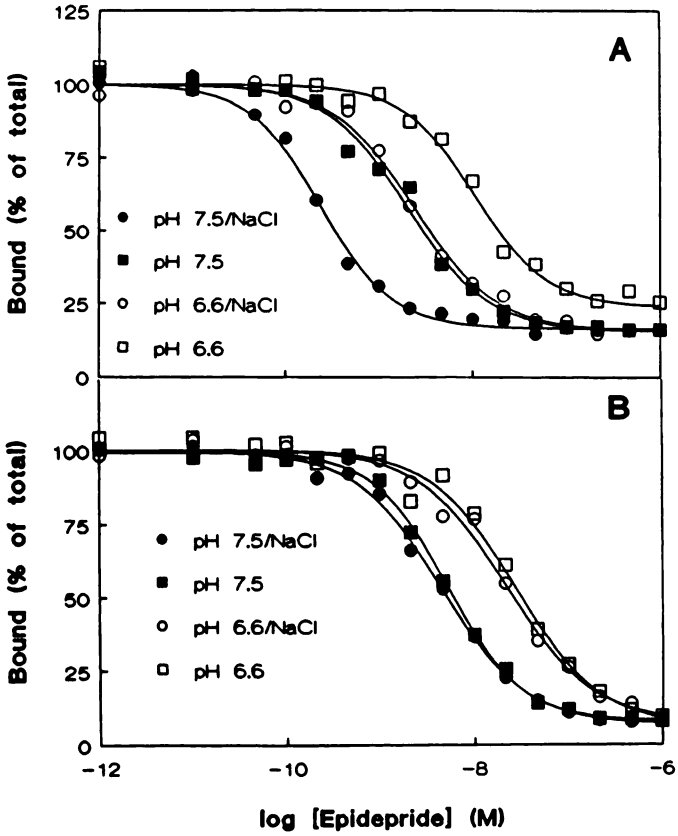

Fig. 2. Inhibition by epidepride of the binding of [^{3}H]spiperone to $D2_{415}$ and Ala-80-D2. Data are shown from a representative experiment in which the binding of 120 pM [^{3}H]spiperone to $D2_{415}$ (A) or Ala-80-D2 (B) was inhibited by increasing concentrations of epidepride. Results are plotted as radioligand bound, expressed as a percentage of total binding in the absence of inhibitor, versus the logarithm of the concentration of epidepride. Assays were carried out at the indicated pH in the presence of 100 mM NMDG-Cl (pH 6.6 and 7.5) or 100 mM NaCl (pH 6.6/NaCl and pH 7.5/NaCl).

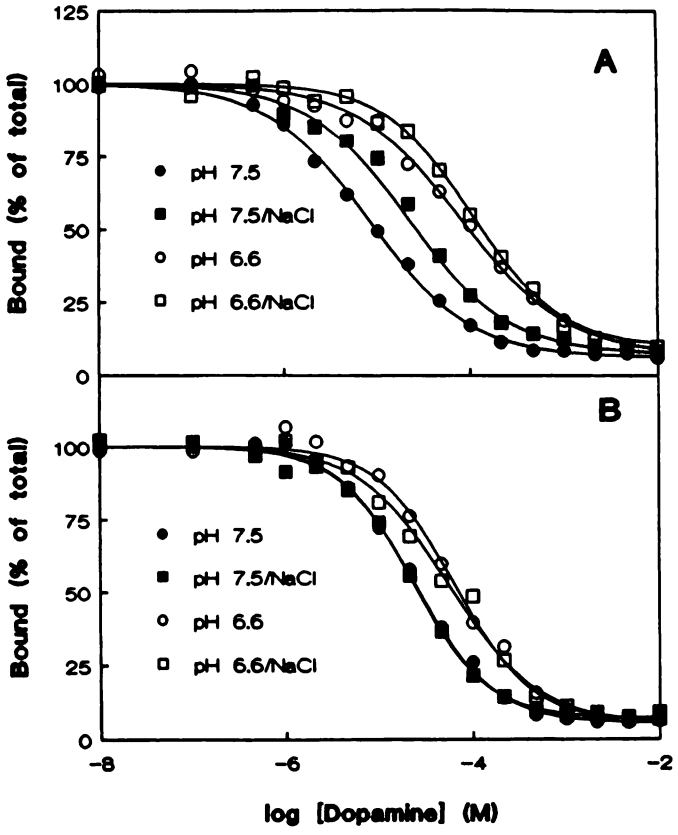

Fig. 3. Inhibition by DA of the binding of [^{3}H]spiperone to $D2_{415}$ and Ala-80-D2. Data are shown from a representative experiment in which the binding of 140 pM [^{3}H]spiperone to $D2_{415}$ (A) or Ala-80-D2 (B) was inhibited by increasing concentrations of DA. Results are plotted as radioligand bound, expressed as a percentage of total binding in the absence of inhibitor, versus the logarithm of the concentration of DA. Assays were carried out at the indicated pH in the presence of 100 mM NMDG-Cl (pH 6.6 and 7.5) or 100 mM NaCl (pH 6.6/NaCl and pH 7.5/NaCl).

Ala-80-D2 (Table 2). Untransfected C_6 cells or cells expressing pRSVneo had no detectable binding of [^{125}I]epidepride (data not shown).

Regulation by [Na$^+$] and pH of the affinity of wild-type and mutant receptors for drugs. One possible cause for the decreased affinity of Glu-80-D2 and Ala-80-D2 for [^{125}I]epidepride, but not [^{3}H]spiperone, was that mutation of Asp-80 altered the regulation of D2 receptors by Na$^+$ or H$^+$. This possibility was evaluated by determination of the apparent affinity of several drugs for inhibiting the binding of [^{3}H]spiperone. [^{3}H]Spiperone was used in these experiments because of its relative insensitivity, compared with [^{125}I]epidepride, to changes in [Na$^+$] or pH between 6.5 and 8.0 (Table 2) (13). The most dramatic effect of substitution of an alanine residue for Asp-80 was a complete loss of the sensitivity of D2 receptors to sodium, as reflected in the lack of a sodium-induced shift in the apparent affinity of the substituted benzamide derivatives epidepride and sulpiride (Fig. 2, Tables 2 and 3) and the agonists NPA and DA (Fig. 3, Tables 2 and 3) for D2 receptors. For example, K_I values for binding of epidepride to $D2_{415}$ in the presence and absence of sodium were 20 and 210 pM, respectively, whereas the corresponding values for binding to Ala-80-D2 were 470 and 570 pM. The same mutation (Ala-80-D2) reduced by half the sensitivity of the binding of all four compounds to lowering of the pH from 7.5 to 6.6. Thus, the K_I

TABLE 3

Shift in wild-type and mutant receptor affinity induced by H$^+$ or Na$^+$

The magnitude of the shift in affinity resulting from variations in pH or [Na$^+$] was determined from the data in Table 2. The free energy change of binding (ΔG^0) was calculated for each assay condition, and the magnitude of the change in ΔG^0 ($\Delta\Delta G^0$) was determined from the absolute value of the difference between two values for ΔG^0. The data in Table 2 yield two values for $\Delta\Delta G^0$ resulting from changes in pH ($\Delta\Delta G^0_{pH}$), one in the presence and one in the absence of NaCl, and two values for $\Delta\Delta G^0$ resulting from changes in [NaCl] ($\Delta\Delta G^0_{NaCl}$), one each at pH 6.6 and 7.5. To simplify, values are shown for the conditions that resulted in the greatest shift in affinity of $D2_{415}$ for each drug. Thus, $\Delta\Delta G^0_{pH}$ shown for binding of epidepride and sulpiride to $D2_{415}$ and each mutant was calculated using affinity values determined in the presence of NaCl, and $\Delta\Delta G^0_{NaCl}$ was calculated from values determined at pH 7.5. For both agonists, the values shown are $\Delta\Delta G^0_{pH}$ in the absence of NaCl and $\Delta\Delta G^0_{NaCl}$ at pH 7.5.

Drug	Variable	Affinity shift		
		$D2_{415}$	Ala-80_{D2}	Glu-80_{D2}
		kcal/mol		
Epidepride	$\Delta\Delta G^0_{pH}$	2.07	1.19	1.52
	$\Delta\Delta G^0_{NaCl}$	1.42	0.12	0.38
Sulpiride	$\Delta\Delta G^0_{pH}$	2.29	1.19	1.48
	$\Delta\Delta G^0_{NaCl}$	1.30	0.15	0.33
NPA	$\Delta\Delta G^0_{pH}$	1.29	0.67	0.50
	$\Delta\Delta G^0_{NaCl}$	0.68	–[a]	0.31
DA	$\Delta\Delta G^0_{pH}$	1.91	1.09	1.11
	$\Delta\Delta G^0_{NaCl}$	0.42	–[a]	–[a]

[a] Absence of a shift in affinity in the direction observed for $D2_{415}$.

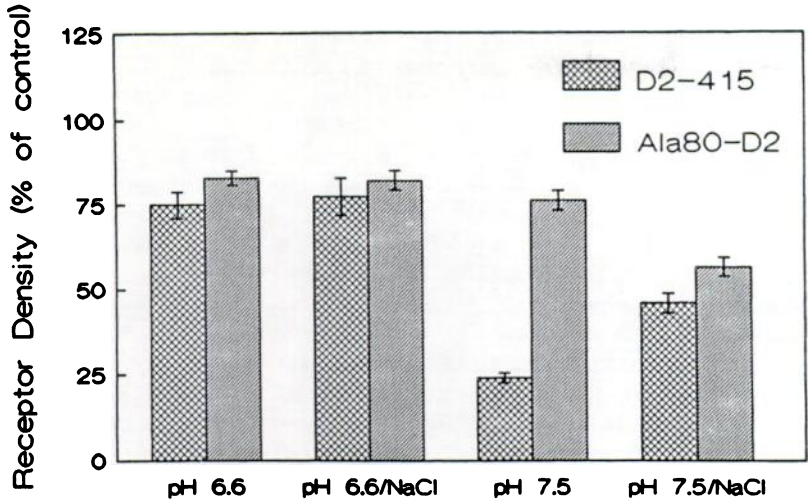

Fig. 4. Inactivation of mutant and wild-type D2 receptors by NEM. Each *bar* represents the mean ± standard error of B_{max} values from six ($D2_{415}$) or seven (Ala-80-D2) experiments in which membranes prepared from C_6 cells expressing the receptors were incubated with 3 mM NEM for 30 min. The density of receptors was then quantified by saturation analysis of the binding of [^{125}I]-epidepride. Data are expressed as a percentage of the control (untreated) density of receptors.

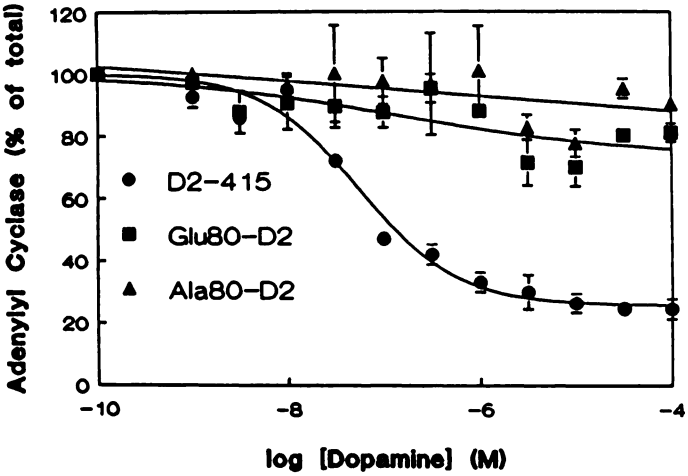

Fig. 5. Inhibition of adenylyl cyclase activity by $D2_{415}$, Glu-80-D2, and Ala-80-D2. Adenylyl cyclase activity was assessed by measurement of the conversion of [^{3}H]ATP to [^{3}H]cAMP in C_6 cells expressing wild-type or mutant D2 receptors. Results shown are mean ± standard error of three (Glu-80-D2, Ala-80-D2) or four ($D2_{415}$) independent experiments, expressed as a percentage of total enzyme activity stimulated by 1 μM isoproterenol in the absence of DA. Total activity was 6.6 ± 0.6% ($D2_{415}$), 6.3 ± 0.7% (Glu-80-D2), and 6.5 ± 0.7% (Ala-80-D2). Basal conversion of [^{3}H]ATP to [^{3}H]cAMP was 0.07%, or approximately 1% of isoproterenol-stimulated enzyme activity.

value for binding of epidepride to $D2_{415}$ was increased 31-fold from 20 to 620 pM, as a result of a decrease of the pH to 6.6, but the K_I value for binding to Ala-80-D2 was increased only 7-fold, from 0.47 to 3.4 nM. Lowering of the pH from 7.5 to 6.6 caused a 24-fold increase in the K_I value for binding of DA to $D2_{415}$, from 0.8 to 19 μM, but the K_I value for binding of DA to Ala-80-D2 was increased only 6-fold, from 1.8 to 11 μM.

Substitution of a glutamate residue for Asp-80 decreased but did not eliminate the regulation by sodium of affinity for epidepride, sulpiride, and NPA, and it also decreased the effect of variations in [H^+] on the binding of the substituted benzamides and agonists (Tables 2 and 3). The K_I value for binding of epidepride to Glu-80-D2, for example, was increased only 2-fold in the absence of sodium, from 0.54 to 1.0 nM, and increased only 12-fold (6.7 nM) by lowered pH. With the exception of two conditions (comparison of the affinity of $D2_{415}$ and Glu-80-D2 for NPA at pH 6.6 or pH 6.6/NaCl), the apparent affinity of agonists for Glu-80-D2 was reduced approximately 10-fold or more, compared with that for $D2_{415}$ or Ala-80-D2. In addition, the affinity of epidepride and sulpiride for Glu-80-D2 tended to be lower than that for $D2_{415}$ or Ala-80-D2 (Table 2).

Inactivation of wild-type and mutant D2 receptors by NEM. At pH 7.5, treatment with 3 mM NEM for 30 min reduced the density of $D2_{415}$ receptors by 76% (Fig. 4). Inactivation of $D2_{415}$ was inhibited by either NaCl (54% decrease) or lowered pH (25% decrease). The density of Ala-80-D2 receptors was decreased only marginally at either pH 6.6 (17%) or pH 7.5 (24%), indicating that the sensitivity of Ala-80-D2 to NEM was not altered by varying [H^+].

Inhibition of adenylyl cyclase activity by wild-type and mutant D2 receptors. In C_6 cells expressing $D2_{415}$, but not in untransfected C_6 cells, DA attenuated isoproterenol-stimulated adenylyl cyclase activity in a concentration-dependent manner. Maximal inhibition was 75% of total enzyme activity, with an EC_{50} for DA of 56 nM (Fig. 5). The mutant Ala-80-D2 was unable to mediate inhibition of adenylyl cyclase activity by DA. The ability of Glu-80-D2 to mediate inhibition of adenylyl cyclase activity was greatly decreased. Maximal inhibition of activity in cells expressing Glu-80-D2 was 25% of total activity, with an EC_{50} of 140 nM (Fig. 5).

Discussion

Asp-80, located in the second transmembrane domain of DA D2 receptors, is strictly conserved among G protein-coupled receptors (26). The data presented here demonstrate that this negatively charged amino acid residue is critical not only for regulation of the conformation of D2 receptors by Na^+ and H^+ but also for coupling of the receptors to at least one signaling pathway.

Two molecular forms of D2 receptors, generated by alternative splicing of a single gene product, have been identified (27–29). The two forms are identical except for a 29-amino acid insert that is present in the third cytoplasmic loop of one form ($D2_{444}$) but absent from the other ($D2_{415}$). The present data demonstrate that [^{125}I]epidepride, like several other D2 receptor ligands (28, 30), did not differentiate between $D2_{415}$ and $D2_{444}$. Furthermore, the affinity of the alternatively spliced forms of D2 receptors for [^{125}I]epidepride was regulated in a similar manner by monovalent cations. As reported previously (13), Na^+ and H^+ had opposing effects on the binding of the substituted benzamide derivative [^{125}I]epidepride. Raising of [Na^+]

increased the affinity of $D2_{415}$ and $D2_{444}$ for [^{125}I]epidepride, whereas lowering of the pH to 6.6 decreased the affinity of both forms of D2 receptors for [^{125}I]epidepride.

Because $D2_{415}$ and $D2_{444}$ did not differ in sensitivity to pH or Na^+, we chose to construct mutants of only one of the forms, $D2_{415}$. Replacement of Asp-80 by an alanine residue (Ala-80-D2) yielded receptors with unchanged affinity for [^{3}H]spiperone but approximately 10-fold lower affinity for [^{125}I]epidepride, compared with wild-type receptors. Mutant receptors in which a glutamate residue was substituted for the aspartate residue (Glu-80-D2) had moderately decreased affinity for [^{125}I]epidepride. The similar affinity of $D2_{415}$, Ala-80-D2, and Glu-80-D2 for [^{3}H]spiperone suggests that substitution of alanine or glutamate for Asp-80 did not result in gross conformational changes. The decreased affinity of Ala-80-D2 and Glu-80-D2 for [^{125}I]-epidepride was apparently related to a decreased sensitivity of the mutant receptors to Na^+ and H^+. Binding of epidepride, sulpiride, DA, and NPA to Ala-80-D2 was completely insensitive to added NaCl, whereas the low pH-induced reduction in ΔG° associated with the binding of the drugs was decreased by 50%. The more conservative substitution of a glutamate residue for Asp-80 also greatly decreased the sensitivity of D2 receptors to Na^+ and H^+, as reflected in the lower magnitude of the ion-induced changes in ΔG°, although the decreased sensitivity tended to be less than observed for Ala-80-D2. One unexpected observation was that the affinity of Glu-80-D2 for the agonists NPA and DA was lower than the affinity of either $D2_{415}$ or Ala-80-D2 for these agonists.

Substituted benzamide drugs are an unusual class of ligands. In rats, substituted benzamides antagonize some of the behavioral effects of stimulation of DA D2 receptors by agonists but are less likely to cause catalepsy than other D2 receptor antagonists (31). Clinically, the compounds are antipsychotic drugs that induce less parkinsonism than classical neuroleptic drugs (32). Substituted benzamide D2 receptor ligands are antagonists *in vitro*, as determined by their ability to prevent inhibition of adenylyl cyclase activity by D2 receptor agonists (2). The extreme sensitivity to pH and Na^+ of the binding of substituted benzamide derivatives, however, distinguishes the drugs from classical antagonists at D2 receptors and from antagonists at other classes of receptors. For example, α_2-adrenergic receptor antagonists are approximately 10-fold less sensitive than agonists to changes in pH or [Na^+] (16). Due to the unusual pharmacological profile of substituted benzamide D2 receptor antagonists, it has been proposed that the compounds label a subclass of DA D2 receptors (31), although *in vitro* data indicate that [^{3}H]spiperone and substituted benzamide ligands label the same population of sites (12, 15, 31, 33). Our data demonstrate that the substituted benzamides differ from classical antagonists such as [^{3}H]spiperone in their mode of binding to D2 receptors, as reflected in the selective effect of mutations of Asp-80 on the binding of epidepride and sulpiride.

Experiments in which the rate of inactivation of DA D2 receptors by NEM depends on [Na^+] and [H^+] have been interpreted as evidence that D2 receptors undergo a Na^+- or H^+-induced conformational change that results in altered affinity of the receptors for substituted benzamide antagonists and agonists (13). If Na^+- and H^+-induced changes in affinity are due to allosteric regulation of the receptor rather than to direct modification of amino acid residues involved in the binding of ligands, mutations that decrease the magnitude of ion-dependent changes in affinity should also decrease the effects of ions on the rate of inactivation of D2 receptors by NEM. NEM-induced inactivation of wild-type $D2_{415}$ is inhibited by NaCl (14, 15) and by lowering of the pH from 7.5 to 6.6 (present results; see also Ref. 13). The most pronounced effect of substitution of alanine for Asp-80 was that, in the absence of NaCl, mutant receptors were inactivated by 30-min treatment with NEM as poorly at pH 7.5 as at pH 6.6. Thus, regardless of pH, Ala-80-D2 appeared to be inactivated to an extent similar to that observed for $D2_{415}$ in the presence of H^+ at pH 6.6. Interestingly, at pH 7.5 the inactivation of Ala-80-D2 was increased by NaCl, indicating that some conformational effects of pH and sodium persist after mutation of Asp-80.

As reported previously, $D2_{415}$ expressed in mammalian cells mediates inhibition of adenylyl cyclase activity by DA (10, 34). The functional coupling of $D2_{415}$ to adenylyl cyclase activity was severely impaired by mutation of Asp-80. We were unable to detect reliable attenuation of enzyme activity by DA in cells expressing Ala-80-D2, whereas DA inhibition of adenylyl cyclase activity in cells expressing Glu-80-D2 was greatly reduced. The density of D2 receptors in the three cell lines differed substantially. This would be unlikely to explain the lack of inhibition of adenylyl cyclase activity by Ala-80-D2, because cells expressing this mutant had a receptor density 5 times that of the cells expressing $D2_{415}$. Although the density of receptors on the cell line expressing Glu-80-D2 (50 fmol/mg of protein) was half that of the cells expressing $D2_{415}$ that were used in the present study, this would not explain the low level of inhibition of adenylyl cyclase activity, because we have detected robust inhibition of enzyme activity in C_6 cells expressing wild-type D2 receptors at a density of 40–60 fmol/mg of protein.[1] The diminished ability of the mutant receptors to inhibit adenylyl cyclase probably results from impaired coupling to G proteins, because our preliminary data indicate that GTP-sensitive binding of DA is abolished or reduced by substitution of alanine or glutamate, respectively, for Asp-80.[1]

An aspartate residue corresponding to Asp-80 apparently serves a pivotal function in the regulation of drug binding and receptor coupling to signaling pathways for a number of neurotransmitter receptors. For β_2-adrenergic receptors, substitution of Asp-79 with an asparagine or alanine residue decreases the affinity of the mutant receptors for agonists, prevents GTP-sensitive interactions of the receptors with G proteins, and reduces isoproterenol-stimulated adenylyl cyclase activity (35, 36). Mutation of Asp-71 of the m_1 muscarinic receptor increases affinity for the agonist carbachol and abolishes stimulation of phosphoinositide hydrolysis (37). Asp-79 of α_{2a}-adrenergic receptors is required for allosteric regulation of the receptors by Na^+ (26). It has been suggested that this conserved residue does not directly interact with ligands or G proteins; rather, the aspartic acid serves to maintain a particular receptor conformation needed for interaction with G proteins and signal transduction (35, 37). Our results support and extend this hypothesis by indicating that the interaction of cations with the aspartate residue modulates receptor conformation.

These data link together, and specify a molecular mechanism for, many phenomena whose relationship to each other was uncertain. Virtually all the effects of sodium on D2 receptors,

[1] K.A.N., R.A.H., B.A.C., A.S. and R.L.N., unpublished observations.

including regulation of receptor conformation and regulation of the affinity of D2 receptors for agonists and substituted benzamide antagonists and perhaps including the effects of sodium on inhibition of adenylyl cyclase activity (2), may be due to an interaction of sodium with a single amino acid residue on D2 receptors. In addition, some of the effects of variations in pH on affinity of D2 receptors for ligands, and on the conformation of D2 receptors, are also mediated by Asp-80. Cations are presumably interacting with the carboxylate side chain of the aspartic acid, but the positioning of the carboxylate anion is apparently crucial in mediating the effects of cations, because substitution with glutamic acid was virtually as debilitating as substitution with alanine. The role of this conserved amino acid residue in both the regulation of receptor conformation by ions and coupling to adenylyl cyclase and other signaling pathways suggests that the effects of these ions on G_i-coupled receptors in particular may be closely linked to mechanisms of signal transduction by the receptors. An interesting future line of investigation will be to determine the ability of Ala-80-D2 and Glu-80-D2 to modulate other signaling pathways, including Na^+/H^+ exchange.

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