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Regulation of Dopamine D2 Receptors by Sodium and pH

KIM A. NEVE

Medical Research Service, VA Medical Center, and Departments of Psychiatry and Pharmacology, Oregon Health Sciences University, Portland, Oregon 97207

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SUMMARY

The role of Na^+ and H^+ in the regulation of D2 receptor affinity for ligands was studied to determine the molecular mechanisms of this phenomenon. The potency of substituted benzamide derivatives and agonists at D2 receptors depended on the concentration of Na^+ and H^+, whereas the potency of other antagonists was relatively unaltered by changes in pH or Na^+ concentration. The potency of agonists was generally decreased in the presence of NaCl or lowered pH. For example, in the absence of sodium the affinity of D2 receptors for dopamine was decreased 17-fold by lowering of the pH from 8.0 to pH 6.8. Addition of NaCl caused 2–4-fold decreases in affinity for most agonists. The affinity of the receptors for two substituted benzamide derivatives, on the other hand, was reduced 6–44-fold by elevated concentrations of H^+ but was enhanced 7–24-fold in the presence of Na^+. The regulation by H^+ of the potency of dopamine was selective for D2 receptors, because binding of dopamine to neostriatal D1 receptors was unaffected by changes in pH. Decreasing of the pH from 8.0 or 7.3 to 6.8 facilitated the dissociation of the substituted benzamide ligand [^{125}I]epidepride from D2 receptors but inhibited dissociation of [^{3}H]spiperone. Furthermore, the presence of NaCl or lowered pH slowed inactivation of D2 receptors by *N*-ethylmaleimide. Together, these data suggest that the conformation of D2 receptors is regulated by both Na^+ and H^+. The affinity of D2 receptors for agonists and substituted benzamide antagonists varies according to the conformational state of the receptors, whereas other antagonists bind to both forms with approximately equal potency. Amiloride is a compound that interacts with many sodium-binding macromolecules. At equilibrium, amiloride inhibited the binding of [^{3}H]spiperone and [^{125}I]epidepride in a manner suggesting a more complex interaction than simple competitive inhibition. The rate of dissociation of both radioligands was enhanced by amiloride, as would be expected for allosteric inhibition of binding. The sensitivity of D2 receptors to pH, sodium, and amiloride may be a reflection of the ability of D2 receptors to modulate Na^+/H^+ exchange.

A fruitful approach to understanding how receptors transfer information has been to investigate agonist-specific characteristics of the interactions of ligands with receptors, on the premise that phenomena specific for agonists are related to the way in which agonists elicit a functional response from receptors. For example, inhibition of the binding of agonists to β-adrenergic receptors by guanine nucleotides suggests that G proteins are involved in the coupling of receptors to adenylyl cyclase (1, 2). Similarly, differences in the energetics of the interactions of agonists and antagonists with β-adrenergic receptors are thought to reflect a conformational change in receptors that results from the binding of agonists (3).

DA D2 receptors belong to a group of receptors that interact with the G protein G_i, to attenuate adenylyl cyclase activity. Although this group of G_i-coupled receptors is defined by effects on one second-messenger system, many of the classes of receptors in this group interact with multiple second messengers. In addition to inhibiting adenylyl cyclase, activation of D2 receptors increases potassium conductance (4) and inhibits calcium channels (5, 6). Furthermore, D2 receptors have recently been shown to modulate Na^+/H^+ exchange in rat anterior pituitary cells (7).

A second characteristic of G_i-coupled receptors is that they are regulated by sodium. Sodium modulates inhibition of adenylyl cyclase (8) and decreases the affinity of G_i-coupled receptors for agonists (9, 10). For α_2-adrenergic receptors, sodium acts directly on the receptor at an intracellular site to alter ligand potency (11, 12). Amiloride, a compound that interacts with membrane proteins having sodium binding sites, allosterically modulates the binding of ligands to α_2-adrenergic receptors (12, 13). Decreased pH also lowers the affinity of the receptors for agonists by direct action on the receptor (12). It seems likely that these effects of amiloride, sodium, and pH reflect the ability of α_2-adrenergic receptors to alter Na^+/H^+ exchange (14), just as regulation of agonist binding by GTP reflects the interaction of receptors with G proteins.

One of the most unusual manifestations of the effects of

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ABBREVIATIONS: G protein, guanine nucleotide-binding protein; DA, dopamine; G_i, inhibitory guanine nucleotide-binding protein; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NEM, *N*-ethylmaleimide; NMDG, *N*-methyl-D-glucamine-H-Cl; 3-PPP, 3-(hydroxyphenyl)-*N*-(1-propyl)piperidine; NPA, propylnorapomorphine.

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sodium on DA D2 receptors is that substituted benzamide antagonists bind with considerably higher affinity in the presence of sodium (15–17). On the other hand, sodium decreases the affinity of D2 receptors for agonists (18, 19). The finding that G proteins do not mediate the sensitivity of D2 receptors to sodium (19, 20) suggests that sodium could be binding to a site on D2 receptors. The experiments in the present report were carried out to define further the mechanisms and significance of the regulation of D2 receptors by sodium and to pursue the similarities between D2 and α_2-adrenergic receptors. My results indicate that the conformational state of DA D2 receptors is regulated by Na^+ and H^+. In addition, amiloride allosterically modulates the binding of ligands to D2 receptors.

Experimental Procedures

Materials. Spiperone, pimozide (Janssen), (+)-3-PPP (Astra), bromocriptine (Sandoz), *cis*-flupenthixol (Lundbeck), and quinpirole (Lilly) were gifts. (*S*)-(−)-*N*-[(1-Ethyl-2-pyrrolidinyl)-methyl]-2,3-dimethoxy-5-(tri-*n*-butyltin)benzamide and unlabeled epidepride were generous gifts from Dr. Tomas de Paulis, Vanderbilt University. (+)-Butaclamol, (*R*)-(−)-NPA, and sulpiride were purchased from Research Biochemicals, Inc. (Natick, MA). $Na^{125}I$ was purchased from Du Pont-New England Nuclear (Boston, MA). [^{3}H]Spiperone (93–112 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). Most other drugs and reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Radioiodination of [^{125}I]epidepride. [^{125}I]Epidepride was prepared essentially as described previously (17), with minor modifications of an iododestannylation reaction analogous to that used for preparation of [^{125}I]iodopride (21). (*S*)-(−)-*N*-[(1-Ethyl-2-pyrrolidinyl)-methyl]-2,3-dimethoxy-5-(tri-*n*-butyltin)benzamide (10 μg/10 μl of absolute ethanol) was mixed with $Na^{125}I$ (5 mCi/10 μl). The solution was acidified by addition of 5 μl of 0.4 N HCl. Chloramine T (10 μg/5 μl of water) was added, and the reaction proceeded for 3 min. The reaction was stopped by addition of sodium metabisulfite (190 μg/10 μl of water). 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) with a mobile phase of 38% ethanol/62% 20 mM potassium phosphate, pH 6.8.

Tissue preparation. Except where indicated, membranes prepared from LZR1 cells were used for these experiments. LZR1 cells, grown as described previously (19), were derived by transfection of a $D2_{415}$ cDNA into mouse L cells (22). 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. Membranes prepared from rat neostriatum were used in some experiments and were prepared as described previously (17).

Radioligand binding assays. 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 6.8 or 8.0, and 100 mM NaCl or 100 mM NMDG (10). (+)-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 2 ml ([^{3}H]spiperone) or 0.5 or 1 ml ([^{125}I]epidepride). Data were analyzed by nonlinear regression, using the program GraphPAD. Competition experiments were carried out in a volume of 1 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 100 to 250 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 either as pK_D (mean ± standard error) or as the geometric means [the antilogarithm of mean logarithms (24)], followed by the 95% confidence interval of the mean in parentheses. Protein was measured by the method of Peterson (25).

The method of Tomlinson and Hnatowich (26) was used to analyze the mechanism by which amiloride inhibits the binding of radioligands to D2 receptors. With this procedure, a family of saturation isotherms is constructed by generation of several curves, each in the presence of a different concentration of inhibitor. If inhibition of radioligand binding is by simple competitive inhibition, the apparent dissociation constant, $K_{D_{app}}$ should increase linearly with [*I*], the concentration of inhibitor, according to the following equation:

$$K_{D_{app}} = K_D(1 + [I]/K_I)$$

where K_D and K_I are the equilibrium dissociation constants for the radioligand and the inhibitor, respectively. Results here are expressed as the ratio $K_{D_{app}}/K_D$, so the right side of the equation is simplified as follows: $K_{D_{app}}/K_D = 1 + [I]/K_I$. Thus, for any given inhibitor at a receptor, the plot of $(K_{D_{app}}/K_D)$ versus [*I*] should be identical regardless of the radioligand used, if the mechanism of interaction is simple competitive inhibition.

Dissociation binding assays. The time course of dissociation of radioligands was determined after incubation of pooled tissue with radioligand for 1 hr in 5 mM K^+-HEPES, pH 7.4, 100 mM NaCl. The concentration of [^{3}H]spiperone in various experiments ranged from 0.3 to 0.5 nM, whereas the concentration of [^{125}I]epidepride ranged from 0.3 to 0.4 nM. The forward reaction was stopped by addition of a saturating concentration (2 μM) of (+)-butaclamol, together with 50 mM K^+-HEPES buffer at the appropriate pH, 100 mM NaCl, and, in some experiments, 0.5 or 1 mM amiloride. The time course of dissociation of specific binding was measured by filtering of single aliquots of tissue at appropriate intervals. Data were analyzed by nonlinear regression, using GraphPAD.

Treatment with NEM. Membrane aliquots were incubated with 3 mM NEM at pH 6.8 or 8.0 (20 mM K^+-HEPES), in the presence or absence of 50 mM NaCl. After 15 or 30 min, 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 7.4). 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. These concentrations had no detectable effect on the binding of [^{125}I]epidepride (data not shown).

Results

Saturation analysis of radioligand binding. There was no detectable effect of Na^+ on the affinity of D2 receptors for [^{3}H]spiperone, a butyrophenone antagonist, whereas lowered pH caused a slight increase in affinity (Fig. 1A, Table 1). B_{max} values, which were not significantly changed by pH or Na^+,

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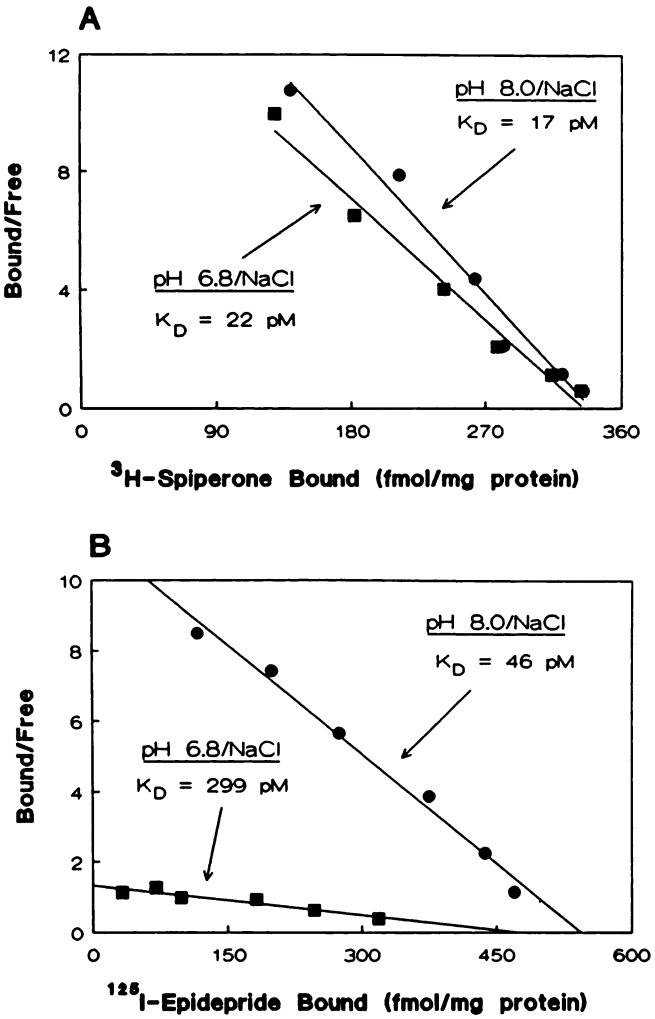

Fig. 1. Regulation by pH of radioligand binding to D2 receptors. Results are shown from one of three independent experiments in which saturation analysis of the binding of [^{3}H]spiperone (A) or [^{125}I]epidepride (B) was carried out at pH 6.8 or 8.0, in the presence of 100 mM NaCl, using membranes prepared from LZR1 cells. The data were transformed and are plotted as (bound radioligand)/(free concentration of radioligand) versus the amount of radioligand bound at each concentration. K_D values shown for each condition were determined by nonlinear regression analysis. The binding of [^{125}I]epidepride was extremely sensitive to changes in pH, whereas the binding of [^{3}H]spiperone was altered only slightly.

TABLE 1

Regulation by Na^+ and pH of the affinity of recombinant D2 receptors for antagonists

Affinity values, expressed as pK_i (mean ± standard error), are given for inhibition of the binding of [^{3}H]spiperone by the indicated drugs, as determined in two or three independent experiments for each drug. Data for spiperone are pK_D values determined from saturation analysis of the binding of [^{3}H]spiperone and were used to convert IC_{50} values to pK_i values for all other drugs.

Drug	pK_i			
	pH 6.8	pH 8.0	pH 6.8/NaCl	pH 8.0/NaCl
Spiperone	10.55 ± 0.02	10.82 ± 0.04	10.62 ± 0.02	10.87 ± 0.06
Pimozide	9.76 ± 0.04	9.78 ± 0.15	9.63 ± 0.09	9.70 ± 0.02
cis-Flupenthixol	9.19 ± 0.18	9.24 ± 0.13	9.18 ± 0.08	9.31 ± 0.06
(+)-Butaclamol	9.75 ± 0.19	9.74 ± 0.12	9.79 ± 0.17	9.66 ± 0.08
Epidepride	7.80 ± 0.08	9.45 ± 0.08	9.17 ± 0.01	10.28 ± 0.04
Sulpiride	6.08 ± 0.10	7.04 ± 0.12	7.06 ± 0.26	7.84 ± 0.06

were 304 ± 25 and 312 ± 15 fmol/mg of protein at pH 6.8 in the presence and absence of 100 mM NaCl, respectively, and 304 ± 21 and 295 ± 15 fmol/mg of protein at pH 8.0 in the presence and absence of NaCl, respectively. In contrast, low pH markedly decreased the affinity of D2 receptors for [^{125}I]-epidepride, a substituted benzamide antagonist, from 46 pM (33–64 pM) at pH 8.0 to 377 pM (296–479 pM) at pH 6.8, with no significant change in B_{max} values (Fig. 1B).

Competition analysis of drug binding. To evaluate further the effects of Na^+ and H^+ on D2 receptors, the potency of drugs for inhibition of the binding of [^{3}H]spiperone was assessed. [^{3}H]Spiperone was used as the radioligand, because its relative insensitivity to pH and sodium simplifies the calculation of apparent affinity values for competing drugs. As observed for spiperone, the potencies of antagonists such as (+)-butaclamol, *cis*-flupenthixol, and pimozide were not affected by changes in pH or NaCl concentration (Table 1, Fig. 2C). Results obtained for epidepride by this method were similar to those determined by saturation analysis of the binding of [^{125}I]epidepride (Fig. 2A, Table 1). The binding of sulpiride, a substituted benzamide, was extremely sensitive to changes in both pH and concentration of NaCl, so that at lower pH or in the absence of NaCl the binding affinity was greatly decreased (Table 1).

The binding of agonists tended to be slightly sensitive to NaCl and moderately sensitive to changes in pH, with decreased potencies resulting from the presence of NaCl or lowered pH. Thus, the apparent affinity of D2 receptors for DA was decreased approximately 2-fold by 100 mM NaCl at pH 8 but decreased 10–20-fold by lowering of the pH from 8.0 to 6.8. The agonists NPA, quinpirole, and (+)-3-PPP displayed 2–6-fold shifts in K_I values as a result of decreasing pH (Fig. 2B, Table 2). The binding of the agonist bromocriptine, however, was unaltered by pH or NaCl concentration.

The effect of decreased pH on the binding of DA to D1 and D2 receptors in rat neostriatum was measured to ascertain whether the effects described above were D2 receptor specific. In the presence of sodium, the apparent affinity of striatal D2 receptors for DA was decreased 10-fold by lowering of the pH from pH 8.0 to 6.8, whereas changes in pH had little effect on the affinity of DA D1 receptors for DA (Table 3). [^{3}H]SCH 23390 bound with higher affinity at pH 8.0 than at pH 6.8 (data not shown), which is consistent with the report of "optimal" binding of the radioligand at pH 8.0 (27).

Effect of pH on dissociation rate. The effect of changes in pH on dissociation of [^{125}I]epidepride was determined after equilibration of 0.3–0.4 nM [^{125}I]epidepride with membranes from LZR1 cells at pH 7.4, in the presence of 100 mM NaCl. Dissociation was initiated by 10-fold dilution with buffer containing 2 μM (+)-butaclamol, 50 mM K^+-HEPES at the appropriate pH, and 100 mM NaCl. As shown in Fig. 3A, the dissociation of [^{125}I]epidepride occurred similarly at pH 7.3 and 8.0, whereas decreasing of the pH to 6.8 accelerated dissociation. Dissociation at pH 7.3 or 8.0 was biphasic, whereas at pH 6.8 the data for dissociation of [^{125}I]epidepride were fit best by assuming only one rate of dissociation (Table 4).

Whereas lowered pH facilitated the dissociation of [^{125}I]-epidepride, dissociation of [^{3}H]spiperone was inhibited at pH 6.8 (Fig. 3B). As observed for [^{125}I]epidepride, dissociation was biphasic at pH 7.3 or 8.0 but monoexponential at pH 6.8 (Table 4).

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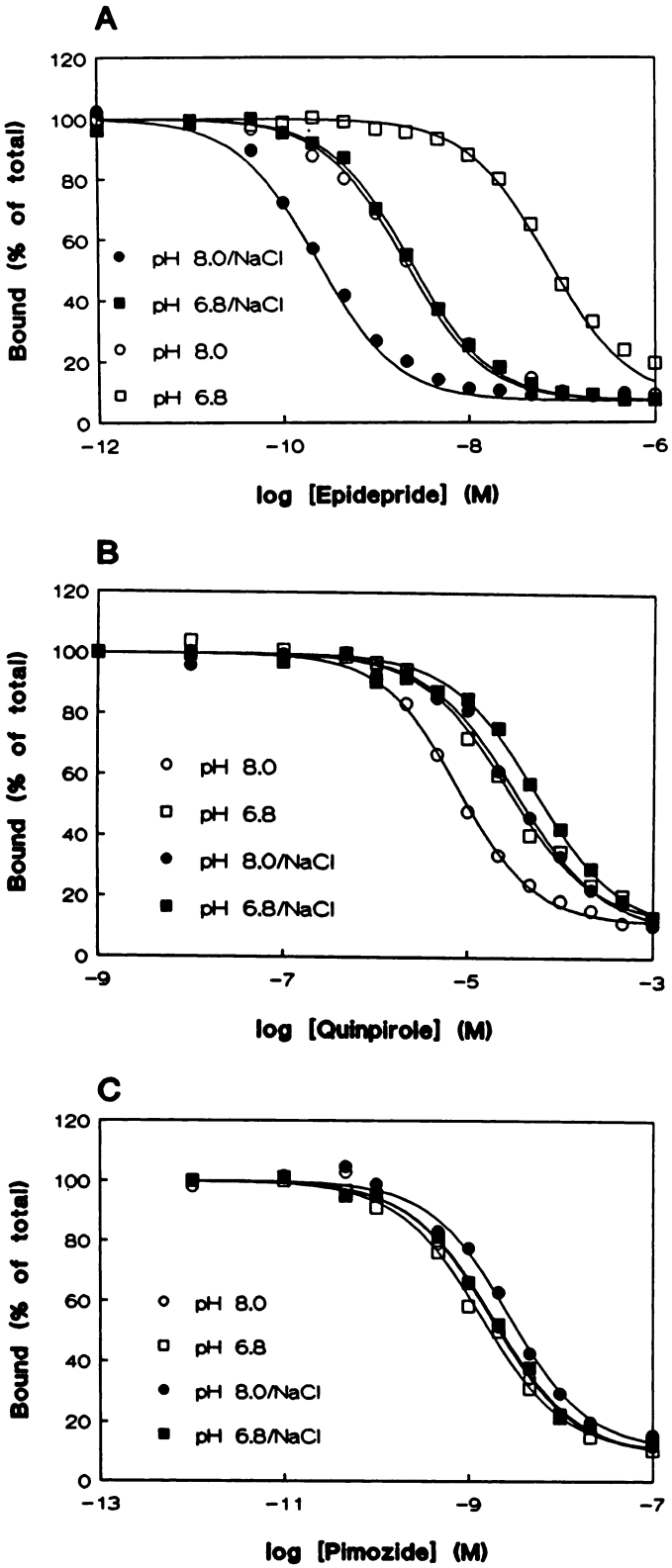

Fig. 2. Effect of Na^+ concentration and pH on drug potency at D2 receptors. Representative experiments are shown in which inhibition of the binding of [^{3}H]spiperone was determined at pH 6.8 or 8.0, in the presence of 100 mM NaCl or 100 mM NMDG, using membranes prepared from LZR1 cells. Data are plotted as radioligand bound, expressed as percentage of total binding in the absence of inhibitor, versus the logarithm of the concentration of inhibitor. Results from all experiments are summarized in Tables 1 and 2. A, The binding of epidepride, a substituted benzamide, was extremely sensitive to changes in H^+ or Na^+ concentrations. The concentration of [^{3}H]spiperone was 0.2 nM. B, The ability of the agonist quinpirole to inhibit the binding of 0.23 nM [^{3}H] spiperone was moderately sensitive to changes in H^+ or Na^+ concentrations. C, The binding of pimozide, an antagonist, was not sensitive to H^+ or Na^+ concentration changes. The concentration of [^{3}H]spiperone was 0.23 nM.

TABLE 2

Regulation by Na^+ and pH of the affinity of recombinant D2 receptors for agonists

Affinity values, expressed as pK_i (mean ± standard error), are given for inhibition of the binding of [^{3}H]spiperone by the indicated drugs, in two to six independent experiments. GTP (100 μM) as well as either 100 mM NaCl or 100 mM NMDG, was included in each assay.

Drug	pK_i			
	pH 6.8	pH 8.0	pH 6.8/NaCl	pH 8.0/NaCl
Bromocriptine	9.10 ± 0.09	9.06 ± 0.05	9.12 ± 0.16	9.16 ± 0.06
NPA	8.24 ± 0.07	8.76 ± 0.13	7.84 ± 0.03	8.15 ± 0.14
Quinpirole	5.60 ± 0.06	6.26 ± 0.02	5.24 ± 0.02	6.69 ± 0.01
DA	4.79 ± 0.09	6.03 ± 0.05	4.89 ± 0.06	5.71 ± 0.02
(+)-3-PPP	4.76 ± 0.06	5.33 ± 0.06	4.45 ± 0.15	5.25 ± 0.10

TABLE 3

Regulation by Na^+ and pH of the affinity of rat neostriatal D1 and D2 receptors for DA

Affinity values, expressed as pK_i from two experiments, are given for inhibition of the binding of [^{3}H]SCH 23390 (D1) and [^{3}H]spiperone (D2) by DA. All assays included 50 mM NaCl and 100 μM GTP.

Receptor subtype	pK_i	
	pH 6.8/NaCl	pH 8.0/NaCl
D1	4.43 ± 0.04	4.58 ± 0.02
D2	3.93 ± 0.25	5.05 ± 0.13

Inactivation of D2 receptors by NEM. At pH 8.0, treatment with 3 mM NEM for 30 min reduced the density of D2 receptors on membranes prepared from LZR1 cells by 81%, from 285 fmol/mg of protein to 57 fmol/mg of protein (Fig. 4). In the presence of 50 mM NaCl, the rate of inactivation of receptors by NEM was decreased, so that only 62% of receptors were inactivated. Treatment with 3 mM NEM for 30 min at pH 6.8 decreased the density of receptors by only 25% in the absence of NaCl and by 17% in the presence of NaCl. K_D values for the binding of [^{125}I]epidepride, carried out at pH 7.4 in the presence of 50 mM NaCl, ranged from 27 to 67 pM in these experiments, with no significant changes in affinity associated with NEM pretreatment. Additional experiments were carried out to ascertain whether the protection against NEM-induced inactivation by high concentrations of H^+ is selective for D2 receptors. The stability of 1 mM NEM at pH 6.8 and 8.0 was monitored spectrophotometrically at 302 nm (28), with no detectable decomposition within 30 min. In addition, the reaction of 1 mM NEM with cysteine occurred rapidly at either pH 6.8 or 8.0, being complete within 15 sec (data not shown).

Amiloride modulation of equilibrium binding of radioligands. Increasing concentrations of amiloride inhibited the binding of either [^{125}I]epidepride or [^{3}H]spiperone, with an IC_{50} value of approximately 0.5 mM (data not shown). A simple competition binding assay is an insensitive method of distinguishing between competitive and allosteric interactions between ligands, so the method of Tomlinson and Hnatowich (26) was used to analyze the modulation by amiloride of radioligand binding to D2 receptors. In this method, saturation analyses of radioligand binding are carried out in the presence of various concentrations of inhibitor. Fig. 5, A and B, shows

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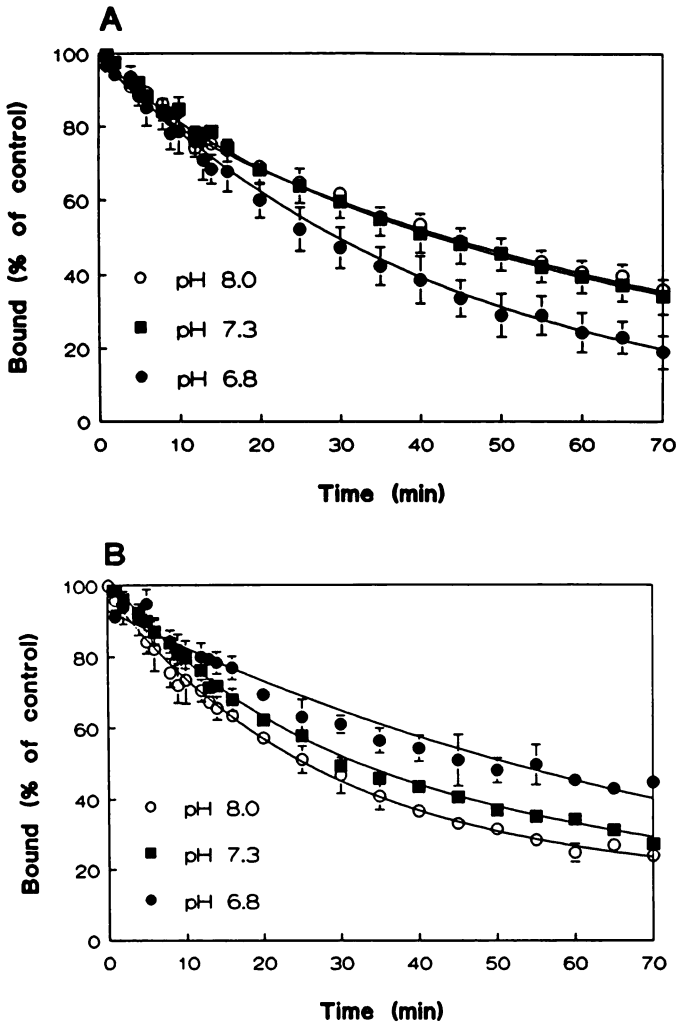

Fig. 3. Effect of pH on dissociation of radioligands from D2 receptors on membranes prepared from LZR1 cells. Averaged data (mean ± standard error) are shown for the time course of dissociation of radioligand in the presence of 100 mM NaCl at the indicated pH. Data are plotted as amount bound, expressed as percentage of specific binding at time 0, versus time. The *curves* are computer-generated mono- or biexponential fits, using the values derived by simultaneous analysis of all experiments for a given condition (Table 4). A, Time course of dissociation of [^{125}I] epidepride (four experiments). At pH 6.8 the radioligand dissociated rapidly and monoexponentially, whereas at pH 7.3 or 8.3 dissociation took place more slowly and biexponentially. B, [^{3}H]Spiperone dissociated rapidly and biexponentially at pH 7.3 or 8.0 (four experiments) but more slowly and monoexponentially at pH 6.8 (three experiments).

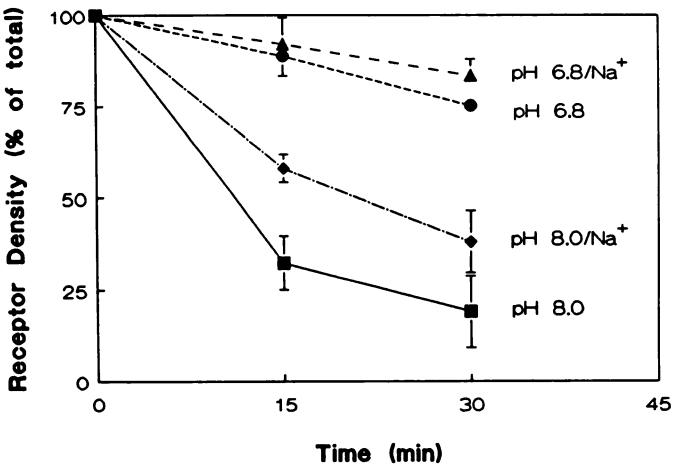

Fig. 4. Sodium- and pH-induced inhibition of D2 receptor inactivation by NEM. Each *point* represents the mean ± standard error of B_{max} values from three experiments, in which membranes prepared from LZR1 cells were incubated with 3 mM NEM for the indicated time before quantification of the density of receptors by saturation analysis of the binding of [^{125}I]epidepride. NEM caused a time-dependent decrease in the density of D2 receptors that was most rapid at pH 8.0 in the absence of NaCl. Inactivation of receptors was inhibited by decreasing of the pH to 6.8 or addition of 50 mM NaCl. Data are plotted as the density of D2 receptors, expressed as percentage of the control (untreated) density, versus the time of incubation with NEM.

the effect of amiloride on saturation curves for binding of [^{125}I]-epidepride and [^{3}H]spiperone, respectively. Amiloride caused a concentration-dependent increase in apparent K_D for both [^{125}I]-epidepride and [^{3}H]spiperone, with no significant change in the apparent density of binding sites. Plots of changes in apparent K_D versus concentration of amiloride demonstrated that the apparent K_D for the binding of [^{3}H]spiperone increased linearly with increasing concentrations of amiloride (Fig. 5C), as would be expected for a reaction in which a drug competitively inhibits the binding of a radioligand. The binding of [^{125}I]epidepride, however, was more sensitive to the presence of amiloride, so that the plot of apparent K_D versus concentration of amiloride deviated upwards (Fig. 5C).

Amiloride modulation of dissociation rate. To explore further the possibility that amiloride allosterically modulates the binding of radioligands to D2 receptors, the effect of amiloride on the rates of dissociation of [^{125}I]epidepride and [^{3}H] spiperone was determined. As displayed in Fig. 6, amiloride caused a concentration-dependent increase in the rates of dissociation of both radioligands. As observed in the absence of

TABLE 4

Rate constants for dissociation of [^{125}I]epidepride and [^{3}H]spiperone from D2 receptors

For each assay condition, data from three or four experiments, as detailed in the legends to Figs. 3 and 6, were analyzed simultaneously using the data analysis program GraphPAD. The rate constants determined by simultaneous analysis are given. For some conditions, the goodness of fit was significantly improved by analysis in terms of two rate constants. If the best fit was a biexponential model, both rate constants are shown, and the proportion of specific binding represented by each constant is given in parentheses. For dissociation of [^{125}I]epidepride at pH 8.0, $p < 0.005$ for improvement of fit by a biexponential model, compared with a monoexponential model. For dissociation of [^{3}H]spiperone in the presence of 0.5 mM amiloride (AMI), $p < 0.01$. For all other biexponential fits, $p < 0.001$.

Radioligand	Rate constant				
	pH 6.8	pH 8.0	pH 7.3	0.5 mM AMI	1.0 mM AMI
			min^{-1}		
[^{125}I]Epidepride (% of total)	0.023	0.157/0.013 (12/88)	0.088/0.013 (14/86)	0.019	0.036/0.011 (65/34)
[^{3}H]Spiperone (% of total)	0.012	0.037/0.003 (22/76)	0.054/0.011 (42/61)	0.074/0.028 (45/56)	0.06

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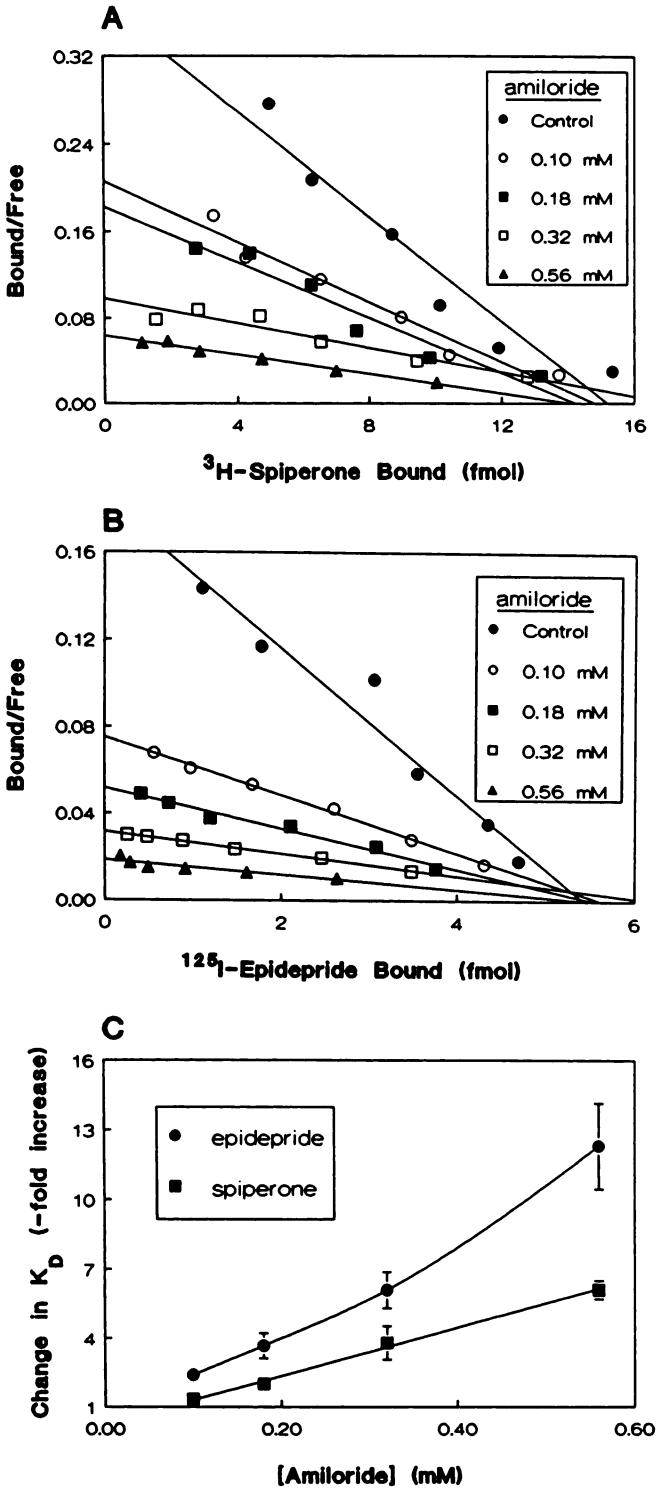

Fig. 5. Inhibition by amiloride of the binding of radioligands to D2 receptors. Results are shown from a representative experiment in which saturation analyses of the binding of [^{3}H]spiperone (A) and [^{125}I]epidepride (B) were carried out in the presence of the indicated concentrations of amiloride, using membranes prepared from LZR1 cells. The data were transformed and are plotted as (bound radioligand)/(free concentration of radioligand) versus the amount of radioligand bound at each concentration. Increasing of the concentration of amiloride increased the apparent K_D for both radioligands, without consistently altering B_{max}. C, The ratio $K_{D_{app}}/K_D$, expressed as the fold increase of the apparent K_D over the K_D value determined in the absence of amiloride, is plotted versus the concentration of amiloride in the assay. The means ± standard errors from three independent experiments are shown.

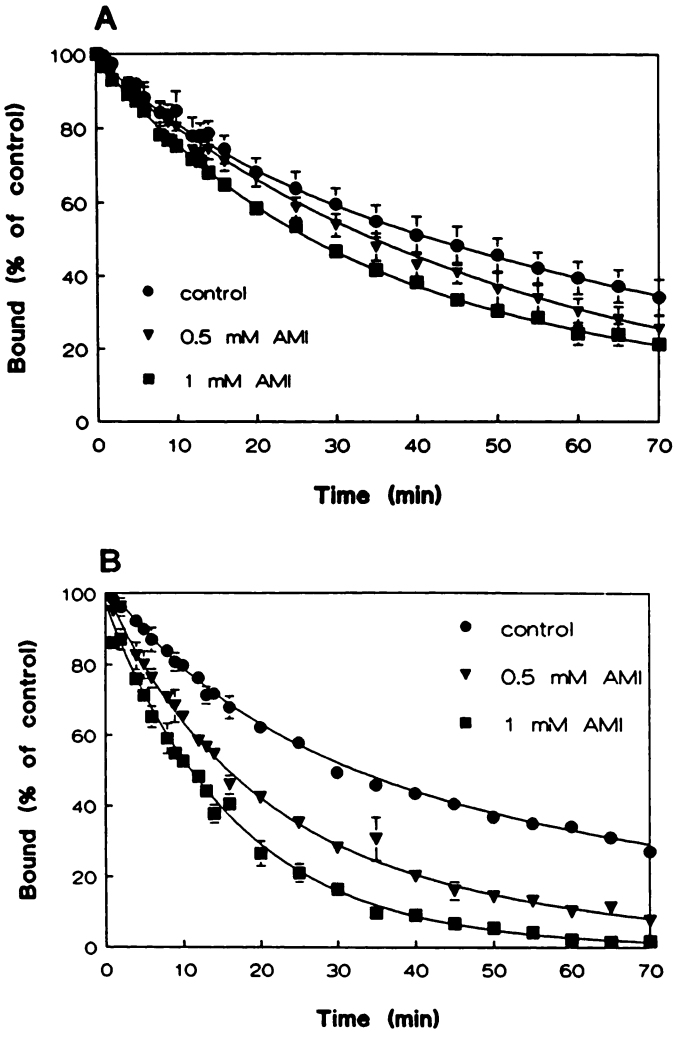

Fig. 6. Effect of amiloride on dissociation of radioligands from D2 receptors on membranes prepared from LZR1 cells. Averaged data (mean ± standard error) are shown for the time course of dissociation of radioligand in the presence of 100 mM NaCl and 0.5 or 1.0 mM amiloride (*AMI*), at pH 7.3. Data are plotted as amount bound, expressed as percentage of specific binding at time 0, versus time. The *curves* are computer-generated mono- or biexponential fits, using the values derived by simultaneous analysis of all experiments for a given condition (Table 4). The control data are the same as those for pH 7.3 in Fig. 3. A, Time course of dissociation of [^{125}I]epidepride (four experiments for control and 0.5 mM amiloride, three experiments for 1 mM amiloride). Dissociation of [^{125}I]epidepride was accelerated by amiloride. B, [^{3}H]Spiperone dissociated more rapidly in the presence of amiloride (four experiments for control and 1 mM amiloride, three experiments for 0.5 mM amiloride).

amiloride, analysis of data for dissociation of [^{125}I]epidepride in the presence of 1 mM amiloride, but not 0.5 mM amiloride, was significantly improved ($p < 0.001$) by assuming two rates of dissociation (Table 4). For [^{3}H]spiperone, the goodness of fit of the data for dissociation in the presence of 0.5 mM amiloride, but not 1 mM amiloride, was significantly improved by assuming two rates of dissociation.

Discussion

The affinity of DA D2 receptors for agonists and substituted benzamide antagonists depended on the concentration of Na^+ and H^+ present in the assay. The binding affinity of all agonists tested, with the exception of bromocriptine, was decreased by

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NaCl or lowered pH. Interestingly, addition of NaCl decreased the affinity of D2 receptors for DA at pH 8.0, but not at pH 6.8. This is analogous to the observation that NaCl decreases the affinity of α_2-adrenergic receptors for agonists more at pH 8.0 than at pH 6.8 (12). The insensitivity of the binding of bromocriptine to cations, together with lack of sensitivity to GTP (19, 29), supports the hypothesis that much of the binding energy of ergopeptines such as bromocriptine is contributed by the peptide side chain (29).

In other experiments, the effect of decreased pH on the binding of DA to rat striatal D1 and D2 receptors was determined. The potency for DA at D2 receptors, but not D1 receptors, was decreased at low pH, indicating that the effect of pH was determined by the class of receptor. Further, these data demonstrate that the effect of pH was not restricted to recombinant D2 receptors or to the $D2_{415}$ form of D2 receptors, because $D2_{444}$ is the predominant form in rat striatum (30). In addition, we have found that the regulation by sodium and pH of binding of [^{125}I]epidepride to recombinant $D2_{415}$ is indistinguishable from regulation of binding to $D2_{444}$ (43).

The binding of the substituted benzamide derivatives epidepride and sulpiride was also sensitive to both Na^+ and H^+. However, whereas both cations decreased the potency of agonists, Na^+ and H^+ had opposing effects on the binding of the substituted benzamides. Na^+ and H^+ had little effect on the binding of the other D2 receptor antagonists tested.

The regulation by Na^+ and H^+ of ligand binding to D2 receptors is similar in many respects to that for α_2-adrenergic receptors (12), with two major differences. First, α_2-adrenergic receptor agonists are more sensitive than D2 receptor agonists to Na^+. Second, the potency of agonists and antagonists at α_2-adrenergic receptors is regulated in an opposite and quantitatively distinct manner by Na^+ and H^+, with agonists generally being approximately 10-fold more sensitive. There is no evidence for a class of ligands for α_2-adrenergic receptors analogous to the substituted benzamides, which are more sensitive than D2 agonists to the concentration of Na^+. Furthermore, the potency of substituted benzamides and agonists at D2 receptors is regulated oppositely by Na^+ but identically by H^+.

Three general mechanisms could result in modulation of ligand binding affinity by Na^+ or H^+, 1) modulation of the conformation or ionization state of the ligand, 2) alteration or ionization of amino acid side chains directly involved in interacting with ligands, and 3) allosteric modulation of the ligand binding site through interaction of Na^+ or H^+ with a separate amino acid side chain. It is unlikely that these results could be explained by modulation of the ligand, for two reasons. First, DA binding to D2 receptors, but not to D1 receptors, is sensitive to pH, implying that D2 receptors, rather than DA, are modulated by pH. Second, most of the sensitivity of D2 receptors to Na^+ and H^+ is abolished by mutation of one amino acid residue, demonstrating that the cations are acting directly on D2 receptors (43).

Two lines of evidence suggest that pH regulates the affinity of D2 receptors for ligands through an allosteric interaction, rather than by alteration of amino acid side chains directly involved in binding to ligands. First, increasing the concentration of H^+ accelerated the dissociation of [^{125}I]epidepride. The rate of dissociation was similar at pH 8.0 and pH 7.3 but changed upon decreasing of the pH to 6.8. The similarity between pH 8.0 and 7.3 is generally true for the pH-dependent phenomena described in this paper, because affinity values determined at pH 8.0 are comparable to those determined previously at pH 7.4 (17, 19). Thus, the effects of changes in pH are mainly associated with the decrease from pH 7.3 to pH 6.8. Whereas lowering of the pH to 6.8 slightly decreased the affinity of D2 receptors for [^{3}H]spiperone, the rate of dissociation of [^{3}H]spiperone was decreased relative to the rates at pH 7.3 and 8.0.

The second line of evidence for an allosteric effect of pH on D2 receptors is the finding that low pH dramatically decreased the rate of inactivation of D2 receptors by NEM. My results also confirm that D2 receptors are inactivated by NEM at concentrations greater than 1 mM (31, 32) and confirm the observation that sodium protects D2 receptors from alkylation by NEM (31, 33). There were several possible explanations for the decreased effectiveness of NEM at pH 6.8, and these alternatives were addressed by monitoring NEM spectrophotometrically. One possibility was that NEM could be unstable at lower pH, but the absorbance of 1 mM NEM at 302 nm was stable at either pH for at least 30 min. A second possibility was that NEM could interact less rapidly with thiols at pH 6.8 than at pH 8.0, but the absorbance of 1 mM NEM was maximally quenched by cysteine within seconds at either pH (data not shown). Inactivation of the receptors occurred much more slowly, implying that the rate-limiting step is something other than the alkylation of the thiol by NEM. The most likely explanation for the present results is that the slow rate of inactivation of D2 receptors is due to steric hindrance and that modifications of the rate of inactivation are due to modifications in steric hindrance as a result of a Na^+- or H^+-induced conformational change in D2 receptors.

Because amiloride has been used to study sodium-binding proteins and the conformation of D2 receptors is regulated by sodium, I also investigated interactions of amiloride with D2 receptors. In equilibrium binding assays, amiloride caused a concentration-dependent decrease in the apparent affinity of D2 receptors for [^{125}I]epidepride and [^{3}H]spiperone, with no change in the density of binding sites. It has been argued that both competitive and noncompetitive inhibitory interactions between two ligands and a receptor will give changes in apparent K_D but not B_{max}, if the binding of both ligands is reversible and at equilibrium (34, 35). Simple competitive inhibition, however, should result in a linear dependence of observed K_D on the concentration of unlabeled inhibitor (26). Indeed, the apparent K_D for the binding of [^{3}H]spiperone increased linearly with the concentration of amiloride. However, expression of the data as the ratio of apparent K_D in the presence of amiloride to control K_D in the absence of amiloride simplifies the equation to: $(K_{D_{app}}/K_D) = 1 + [I]/K_I$. According to this equation, the plot of $K_{D_{app}}/K_D$ versus [amiloride] should be identical for any D2 receptor radioligand under the following conditions: 1) each radioligand binds to the same site in a simple bimolecular reaction, according to the law of mass action, and 2) inhibition of binding of each radioligand by amiloride is a simple competitive interaction.

The curves resulting from inhibition of the binding of [^{125}I]-epidepride and [^{3}H]spiperone by amiloride were quite different, which is consistent with other data indicating that the conditions described above do not apply. The complicated kinetics for binding of [^{125}I]epidepride in the presence of sodium (17) suggest that the first condition is not met. In addition, the

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finding that amiloride allosterically accelerates the dissociation of both [^{3}H]spiperone and [^{125}I]epidepride from D2 receptors indicates that the second condition, simple competitive inhibition by amiloride, does not apply. Although the type of interaction between amiloride and the D2 receptor radioligands cannot be determined from these data, the results do not fit those expected for hyperbolic competitive inhibition (26), in which the inhibitor modulates the affinity of the receptor for the radioligand solely through binding to an allosteric site. It seems likely that, as at α_2-adrenergic receptors (13), amiloride inhibits the binding of radioligands to D2 receptors both competitively and allosterically.

These results indicate that Na^+ and H^+ regulate the conformation of DA D2 receptors. The equilibrium binding affinity of most antagonists is similar for either conformation, whereas the affinity of D2 receptors for agonists and substituted benzamide antagonists varies according to the conformational state. The precise relationship between the allosteric effects of Na^+, H^+, and amiloride is not clear, but three lines of evidence indicate that the cations are acting similarly. First, the binding of a drug to D2 receptors was, in general, sensitive to both Na^+ and H^+ or to neither. Second, both Na^+ and H^+ caused a conformational change that protected against akylation by NEM. Finally, mutation of one strictly conserved amino acid residue of D2 receptors, Asp-80, abolished sensitivity of the receptors to Na^+ and greatly reduced sensitivity to pH (43).

The regulation of the potency of D2 receptor ligands by Na^+ and H^+ is specific for agonists and substituted benzamide antagonists. As an agonist-selective phenomenon, it may be related to the way in which D2 receptors function as transducers. The exchange of Na^+ and H^+ is regulated by several neurotransmitters, including DA D2 receptors, and appears to be involved in hormonal control of some secretory responses (7, 14, 36). One possibility is that, as has been proposed for α_2-adrenergic receptors (12), regulation of D2 receptors by Na^+, H^+, and amiloride is a manifestation of the ability of the receptors to modulate Na^+/H^+ exchange.

Although substituted benzamides are antagonists, as assessed behaviorally (37) and by blockade of DA-inhibited adenylyl cyclase (38), the sensitivity of this family of drugs to Na^+ and pH makes it unique among ligands for D2 and other receptors. Substituted benzamide antagonists are less likely than other D2 receptor antagonists to cause catalepsy in rats (37). The characteristics of the binding of substituted benzamides *in vitro* may be related to their behavioral specificity.

The data presented here do not differentiate between the possibility that changes in pH induce a conformational change due to general effects of altered ionization of a number of amino acid side chains and the possibility that Na^+ and H^+ bind to one site on D2 receptors to regulate the conformational state. The latter possibility is supported by the finding that, for α_2-adrenergic receptors, mutation of one amino acid residue, Asp-79, yields a receptor whose affinity for ligands is no longer sensitive to Na^+ (39). We have found that the corresponding amino acid residue in D2 receptors (Asp-80) confers sensitivity to pH and Na^+ on the binding of ligands to D2 receptors (43).

Asp-80, as well as its corresponding residue in other receptors, is critical not only for regulation of receptors by ions but also for coupling of receptors to several signaling pathways involving G proteins, including inhibition (43) and stimulation (40, 41) of adenylyl cyclase and stimulation of polyphosphoinositide hydrolysis (42). It has been hypothesized that this conserved amino acid residue is involved in maintaining a receptor conformation that permits modulation of signaling pathways by agonists (40, 42). This hypothesis agrees well with our results, which also suggest that, for some receptors, it is the appropriate interaction of Na^+ and H^+ with the conserved aspartate residue that maintains a functional receptor conformation.

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Send reprint requests to: Kim A. Neve, Medical Research Service (151LL), VA Medical Center, 3710 SW US Veterans Hospital Rd., Portland, OR 97207.

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