Biochemical Pharmacology, Vol. 40, No. 8, pp. 1739–1746, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

EFFECTS OF MONOVALENT CATIONS ON NEOSTRIATAL DOPAMINE D_2 RECEPTORS LABELED WITH [^{3}H]RACLOPRIDE

TOMÁS A. READER,* SYLVIE BOULIANNE, EDUARDO MOLINA-HOLGADO and KAREN M. DEWAR

Centre de recherche en sciences neurologiques, Département de physiologie, Université de Montréal, Montréal, Québec H3C 3J7, Canada

(*Received* 2 *January* 1990; *accepted* 9 *April* 1990)

Abstract—Specific [^{3}H]raclopride binding to dopamine D_2 receptors in the rabbit neostriatum was investigated in the presence of the monovalent cations sodium, lithium and potassium. NaCl and LiCl produced concentration-dependent elevations in specific [^{3}H]raclopride binding with sodium inducing approximately 50% more binding than lithium. Inhibition of [^{3}H]raclopride binding by the antagonist (+)-butaclamol was unaffected by the presence of sodium or lithium in the incubation medium. In contrast, the potency of dopamine to compete with [^{3}H]raclopride was decreased by these two ions. This effect was more pronounced in the presence of sodium than lithium and was observed for both the high- and low-affinity states of the D_2 receptor. The guanine nucleotide derivative 5′-guanylylimidodiphosphate (Gpp(NH)p) reduced the potency of dopamine to compete with [^{3}H]raclopride binding in both the presence and absence of cations; however, this effect of Gpp(NH)p was a shift of the D_2 receptors from a high to a lower affinity state. Saturation binding curves in the presence of sodium or lithium were compared with experiments carried out in the absence of monovalent cations (sucrose) and demonstrated that these ions increased the affinity (judged by the equilibrium dissociation constant K_d) of the neostriatal [^{3}H]raclopride binding sites. While NaCl produced a significantly greater change in the K_d of [^{3}H]raclopride binding as compared to LiCl, no differences were apparent in the maximum binding capacity (B_{max}) values determined in the presence of these two cations. In conclusion, the results indicate that [^{3}H]raclopride binding to rabbit neostriatal membranes exhibits a sensitivity to monovalent cations that is consistent with the ionic regulatory properties of the D_2 receptor. Moreover, although lithium and sodium influence specific [^{3}H]raclopride binding in a similar manner, there appear to be quantitative differences between these two ions.

Dopamine receptors have been classified into two distinct categories by virtue of their ability to interact with adenylate cyclase [1]. Receptors that are coupled to this enzyme modulate cyclic (cAMP) AMP formation by interacting with a stimulatory (G_s) or an inhibitory (G_i) guanine nucleotide binding protein. Activation of the D_2 class of receptors is known to inhibit adenylate cyclase [2], and this response appears to be mediated via a G_i protein, the coupling of which can be inhibited by pertussis toxin [3]. While the guanine nucleotides modulate the binding of ligands to receptor sites that function to inhibit or activate this enzyme, sodium (Na^+) is involved in the regulation of receptors that are either unassociated with or negatively coupled to adenylate cyclase [4, 5]. It has also been established that D_2 receptors exist in two affinity states of approximately equal proportion [6, 7]. Receptors that are coupled to G proteins exhibit high affinity (D_2^{high}) for the agonist, whereas those receptors in the low-affinity state (D_2^{low}) are believed to be dissociated from the nucleotide-binding protein [8, 9]. Antagonist ligands can bind with equal affinity to the two states of the D_2 receptor, while agonist drugs can be used to discriminate between these two sites. It has been proposed that Na^+ and guanine nucleotides decrease the affinity of agonist binding to the D_2 receptor primarily through the dissociation of the G protein from the receptor, resulting in the transition from the D_2^{high} state to a low-affinity (D_2^{low}) conformation of the active recognition binding site [9–11]. Replacement of Na^+ by lithium (Li^+) was also found to elicit this effect [11]. Structurally different types of D_2 antagonists may display a differential regulation by Na^+, and the binding of the benzamide series of compounds to the D_2 receptor has been shown to be sodium dependent [12, 13].

The aim of this study was to examine the regulatory effects of monovalent cations (Na^+, Li^+ and K^+) and the non-hydrolyzable guanine nucleotide derivative 5′-guanylylimidodiphosphate (Gpp(NH)p) on the binding properties of the novel benzamide [^{3}H]raclopride to D_2 receptors [14–16] using membranes from the rabbit neostriatum. In addition, the interactions of agonists and antagonists with the D_2 receptor labeled with [^{3}H]raclopride were studied (inhibition curves) under different ionic conditions. Finally, the kinetic properties of [^{3}H]raclopride binding were also examined to determine the "on" and "off" rates in the presence of either sodium or lithium ions.

MATERIALS AND METHODS

The radioligand [*methoxy*-^{3}H]raclopride HCl (sp. act. 61.5 Ci/mmol) was purchased from Dupont,

* Correspondence: Dr. Tomás A. Reader, Département de physiologie, Université de Montréal, CP 6128 Succursale A, Montréal, Québec H3C 3J7, Canada.

Boston, MA; the scintillation fluid (Betamax™) from ICN Radiochemicals, Irvine, CA; (±)-sulpiride HCl from the Fisher Scientific Co., Fair Lawn, NJ; and (+)-butaclamol HCl from Research Biochemicals Inc., Natick, MA. Dopamine HCl, Tris-[hydroxymethyl]-aminomethane (Tris), NaCl, KCl, LiCl, 5′-guanylylimidodiphosphate sodium salt and sucrose were obtained from the Sigma Chemical Co., St. Louis, MO.

Adult male albino New Zealand rabbits (1.5–2.0 kg; La Ferme Lapro Inc., Stukeley Sud Québec) were decapitated with a guillotine and their brains quickly removed and placed on ice. A series of 1.0 to 1.5 mm thick sections (usually 3–4) were cut from each brain on a cold plate, and the neostriatum (caudate and putamen) was dissected out. The tissue samples were homogenized with a Tissumizer™ (Tekmar Co., OH) in 40–100 vol. (w/v) of ice-cold Tris–Cl buffer (50 mM at pH 7.4) and centrifuged at 20,000 *g* for 10 min at 4°. The pellets, after one wash by suspension and recentrifugation, were resuspended in Tris–Cl (50 mM, pH 7.4) buffer.

For the binding of [^{3}H]raclopride to the membrane homogenates, all drugs and the radioligand were made up in Tris–Cl (50 mM, pH 7.4). To avoid oxidation of dopamine, the first dilution was in 0.1% (w/v) ascorbic acid. The binding experiments were initiated by the addition of the radioligand in 100-μL aliquots to tubes already containing 300 μL of the membrane preparation, 100 μL of Tris–Cl buffer and 500 μL of Tris–Cl buffer (pH 7.4) containing 10 mM KCl plus a 240 mM concentration of either NaCl, LiCl or sucrose; the final assay volumes were 1 mL. After incubating for 45 min at 25°, binding was assessed by rapid filtration (<5 sec) over Whatman GF/C glass fiber filters, followed by two washes (<10 sec) with 5 mL of cold buffer. Radioactivity was determined by liquid scintillation spectrometry in an LKB Rackbeta™ II counter (efficiency 50–60%). Specific binding was defined as the total binding minus the nonspecific counts obtained in the presence of a 300 μM concentration of the D_2 antagonist (±)-sulpiride [15, 17]. Protein concentrations determined [18] in 100-μL aliquots of the membrane preparation were, in the final incubations, between 0.05 and 0.20 mg/mL.

The results are expressed as means ±SE. The statistical significance of the differences between two samples was evaluated with the Student's *t*-test. When more than one comparison was made, the significance of differences among samples was evaluated by one-way analysis of variance (ANOVA). Only probability (P) values smaller than 0.05 were considered to be significant. Model testing of the binding data was achieved using LIGAND, i.e. a weighted nonlinear least-squares curve-fitting program [19, 20], and the choice of the model that best fit the experimental data was achieved using the appropriate *F* test.

RESULTS

Influence of monovalent cations on [^{3}H]*raclopride.* The binding of [^{3}H]raclopride to membrane preparations from the rabbit neostriatum was studied in the presence of increasing concentrations (0–150 mM) of NaCl, LiCl or KCl in 50 mM Tris–HCl buffer (pH 7.4). The osmolarity of the different solutions was maintained at 300 mOsM by the addition of appropriate concentrations of sucrose to the medium. The experiments illustrated in Fig. 1 demonstrate the sensitivity of specific [^{3}H]raclopride binding to NaCl, LiCl or KCl. Both Na^+ and Li^+ produced concentration-dependent elevations in specific [^{3}H]raclopride binding with maximal binding obtained above 30 mM and maintained up to 120 mM (ED_{50} = 3.12 ± 0.74 and 4.06 ± 0.99 mM in Na^+ and Li^+ respectively). The maximal binding in the presence of 120 mM NaCl was approximately 2-fold higher than that obtained in 120 mM LiCl (i.e. 6820 ± 1400 dpm/mg protein in sodium versus 3860 ± 670 dpm/mg protein in lithium). In contrast, the addition of KCl to the incubation medium did not substantially affect specific [^{3}H]raclopride binding. The small elevations of specific binding above control (250 mM sucrose; 1710 ± 160 dpm/mg protein) observed with concentrations of 100 and 120 mM KCl (2310 ± 530 and 2360 ± 450 dpm/mg protein) were not statistically significant.

Effects of monovalent cations on the equilibrium binding parameters of [^{3}H]*raclopride.* Saturation binding isotherms of [^{3}H]raclopride to membrane preparations from neostriatum were performed at equilibrium (45 min at 25°) with ten concentrations (0.05 to 10 nM) of the radioligand in Tris–HCl buffer containing a 120 mM concentration of either NaCl, LiCl or sucrose, i.e. absence of cations. In the presence of NaCl and LiCl, specific binding was saturable and of high affinity (K_d values of 0.99 ± 0.13 and 2.37 ± 0.24 nM in the presence of NaCl and LiCl respectively), and the Scatchard analysis [21] indicated one single apparent class of binding sites with no evidence of cooperativity. The binding of [^{3}H]raclopride in the absence of ions was not saturable at the highest concentration of radioligand employed with an apparent dissociation constant (K_d) of around 6.41 ± 0.97 nM. The addition of monovalent cations to the incubation buffer increased the affinity of [^{3}H]raclopride for the striatal D_2 receptor, as evidenced by the lower K_d values (Table 1). However, the addition of Na^+ to the buffer elicited a significantly greater change in the affinity of [^{3}H]raclopride for the D_2 receptor than the addition of Li^+. These observations also suggest that the cations increased the density of binding sites above control values; however, since binding in the absence of ions was not saturable at the highest concentration of ligand tested, the estimate of B_{max} obtained from the Scatchard analysis in sucrose buffer (254 ± 37 fmol/mg protein) may not reflect accurately the real density of D_2 receptors. There was no significant difference between the B_{max} values of [^{3}H]raclopride binding in buffer containing NaCl (448 ± 29.7 fmol/mg protein) or LiCl (400 ± 32.4 fmol/mg protein).

Kinetics of [^{3}H]*raclopride binding in the presence of* NaCl *or* LiCl. The association of [^{3}H]raclopride (1 nM) in Tris–HCl buffer containing either NaCl or LiCl at 25° was rapid and reached a steady state within 15 min (Fig. 2A). In both incubation buffers specific binding was stable for at least 2 hr at 25°, indicating that neither binding sites nor radioligand

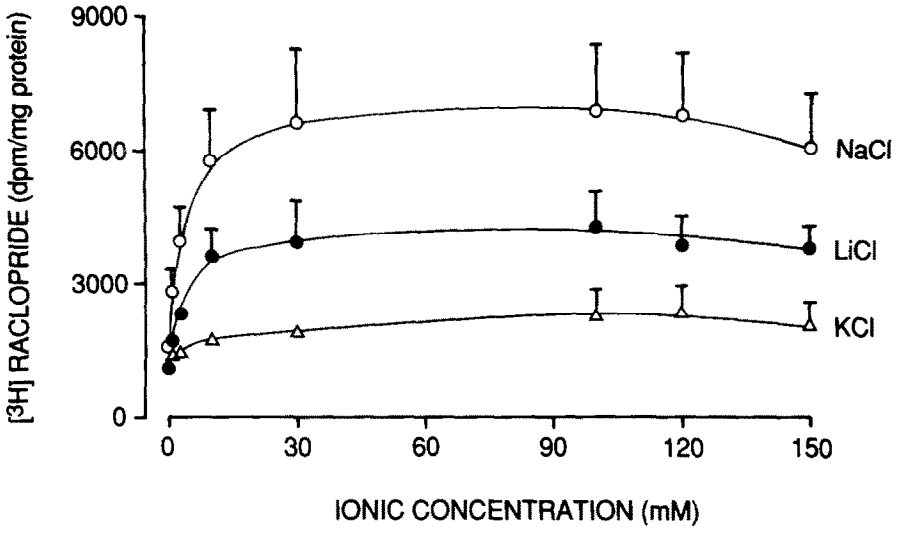

Fig. 1. Effect of monovalent cations on specific [^{3}H]raclopride binding to membrane preparations from rabbit neostriatum. The incubation medium was made of 50 mM Tris–Cl buffer (pH 7.4) plus increasing concentrations of either NaCl, LiCl or KCl, and the osmolarity was maintained at 300 mOsM by the addition of appropriate amounts of sucrose. The incubations were started by the addition of 100 μL of [^{3}H]raclopride (1 nM final concentration) and proceeded for 45 min at 25° (final volume 1 mL). Data points are the means (±SE) of four separate experiments, each conducted in triplicate. Nonspecific binding, defined as the counts in the presence of 300 μM (±)-sulpiride, was subtracted from the curves. In additional experiments performed only in 50 mM Tris–Cl and sucrose (data not shown), specific binding was as low as in KCl.

Table 1. Parameters of specific [^{3}H]raclopride binding to membranes from rabbit neostriatum in buffers with different ionic composition

	N	K_d (nM)	B_{max} (fmol/mg protein)
NaCl	10	0.986 ± 0.129*†	448 ± 29.7†
LiCl	10	2.374 ± 0.236‡	400 ± 32.4‡
Sucrose	9	6.410 ± 0.969	254 ± 37.1

The equilibrium dissociation constants (K_d) and the densities (B_{max}) of specific [^{3}H]raclopride binding sites were determined [21] with ten radioligand concentrations (0.05 to 10 nM). Values are means ±SE of 9–10 (N) independent experiments with separate membrane preparations, all performed in duplicate, i.e. two tubes for total binding and two tubes for nonspecific counts. In all cases, nonspecific binding was determined as the counts in the presence of 300 μM (±)-sulpiride. The incubation medium was Tris–Cl buffer (50 mM at pH 7.4) with 5 mM KCl containing either 120 mM NaCl, 120 mM LiCl or 120 mM sucrose.

*–‡ Significant differences were determined by one-way analysis of variance (ANOVA): *$P < 0.05$ between "NaCl" and "LiCl"; †$P < 0.001$ between "NaCl" and "Sucrose", and ‡$P < 0.01$ between "LiCl" and "Sucrose."

degraded during the incubation time. The association of [^{3}H]raclopride was more rapid in the presence of lithium ($k_{+1} = 0.056\ min^{-1}\ nM^{-1}$) than in sodium ($k_{+1} = 0.022\ min^{-1}\ nM^{-1}$). After equilibrium binding was attained (30 min), the dissociation kinetics were examined by the addition of 1 μM (+)-butaclamol to prevent the reassociation. The dissociations were rapid at 25° with half-time displacement ($T_{1/2}$) values of 7.27 and 7.19 min in NaCl and LiCl respectively. After 30 min the dissociation of [^{3}H]raclopride was almost complete, i.e. more than 90% specific binding was displaced (Fig. 2A) and the dissociation rate constants at this ligand concentration (1 nM) were 0.095 min^{-1} and 0.0963 min^{-1} for NaCl and LiCl respectively. The association and dissociation rates of [^{3}H]raclopride were also calculated through the measurement of association using various concentrations of radioligand (0.5 to 3 nM). The linear plot of the k_{obs} of association versus the radioligand concentration provided estimates of the k_{-1} at the "Y" intercept and of the k_{+1} from the slope (Fig. 2B). From the calculation of this plot the association and dissociation constants of [^{3}H]raclopride binding in the presence of NaCl ($k_{+1} = 0.0322\ min^{-1}\ nM^{-1}$; $k_{-1} = 0.0827\ min^{-1}$) were slower than in LiCl ($k_{+1} = 0.0419\ min^{-1}\ nM^{-1}$; $k_{-1} = 0.1255\ min^{-1}$). The K_d values for [^{3}H]raclopride calculated from the ratio k_{-1}/k_{+1} were 2.56 and 2.99 nM for Na^+ and Li^+ respectively.

Effects of monovalent cations on the competition of [^{3}H]raclopride binding by dopamine and (+)-butaclamol. The effects of NaCl and LiCl on the affinities of the dopaminergic antagonist (+)-butaclamol and the natural substrate dopamine for the D_2 receptor labeled by [^{3}H]raclopride were studied in competition experiments. The inhibition of specific [^{3}H]raclopride binding by (+)-butaclamol was not affected significantly by the addition of either NaCl or LiCl to the incubation buffer, and the IC_{50} values were 6–9 nM. These competition curves were steep, with Hill coefficients (n_H) of about 1 (Table 2). In contrast, the potency of dopamine to compete with [^{3}H]raclopride was reduced significantly by these two ions (Table 2). The competition curves of dopamine in the absence of ions were shallow with an n_H value of 0.56 ± 0.05

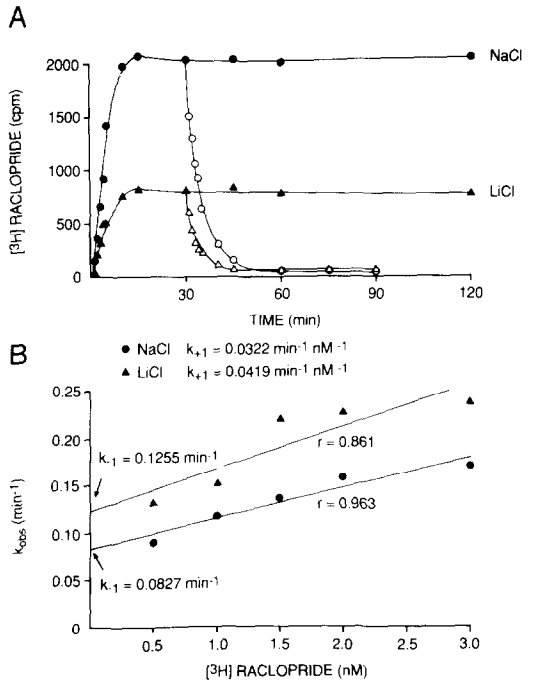

Fig. 2. Association and dissociation kinetics of specific [^{3}H]raclopride binding to membrane preparations from rabbit neostriatum in 50 mM Tris–Cl buffer (pH 7.4) with 5 mM KCl containing either 120 mM NaCl or 120 mM LiCl. (A) For the measurement of the association kinetics, the membrane preparations (200-μL aliquots) were incubated at 25° in the presence of [^{3}H]raclopride (added in 100-μL aliquots) for increasing periods of time before filtration. Nonspecific binding was defined as the counts in the presence of 300 μM (±)-sulpiride. The final incubation volumes were 500 μL. To determine the dissociation rate, the membrane preparations were incubated for 30 min to achieve equilibrium, and specifically bound [^{3}H]raclopride was then measured at increasing times after the addition of 500 μL of buffer containing unlabeled (+)-butaclamol (1 μM final concentration; final incubation volume, 1 mL). The curves shown in (A) were obtained using a 1 nM concentration of [^{3}H]raclopride. (B) The kinetic studies were conducted at five radioligand concentrations (0.5 to 3 nM), and the k_{obs} was determined for every concentration. The association rate constant (k_{+1}) was the slope of k_{obs} versus ligand concentration, and the incercept gave the dissociation rate constant (k_{-1}).

and could be described by a two-site model with around 60% of binding to a high-affinity site (D_2^{high}) and 40% to a low-affinity site (D_2^{low}). The addition of ions to the buffer did not alter the n_H values for dopamine (0.53 ± 0.03 and 0.50 ± 0.02 with NaCl and LiCl respectively). In fact, the presence of Na^+ or Li^+ diminished the affinity of dopamine for both the D_2^{high} and the D_2^{low} states of the D_2 receptor (Table 3), whereas neither cation significantly altered the relative proportions ($\%R_H$ and $\%R_L$) of these sites. The change in the affinity of the D_2 receptor for dopamine was more pronounced in the presence of sodium than in lithium.

Effects of guanine nucleotides on the competition of [^{3}H]raclopride by dopamine. Since guanine nucleotides have been shown to modulate the binding of agonists to D_2 receptors, the effect of 100 μM Gpp(NH)p was examined on dopamine competition curves for [^{3}H]raclopride in rabbit neostriatum. If the buffer only contained sucrose, the guanine nucleotide Gpp(NH)p did not influence significantly the competition of dopamine for [^{3}H]raclopride binding sites (Table 4). The curves, however, were shallow with Hill coefficients of less than one (about 0.5) and could be again best described by a two-site model. When analyzed for a two-site binding model, Gpp(NH)p (100 μM) did not alter significantly either the proportion of D_2^{high} and D_2^{low} states of the D_2 receptor or the affinity of dopamine for these sites (Table 5). In contrast, the addition of Gpp(NH)p to buffer containing 120 mM NaCl or LiCl significantly reduced the potency of dopamine to compete for the D_2 receptor labeled with [^{3}H]raclopride. Interestingly, the Hill coefficients of these competition curves in Gpp(NH)p were increased to 0.62 and 0.64 (Table 4). The analysis of these data assuming a two-site model indicated that the presence of Gpp(NH)p resulted in a decrease in the percentage of high-affinity (R_H) sites (a shift from 60 to 30%), whereas the affinities of the remaining D_2^{high} and D_2^{low} sites did not change (Table 5). These findings indicate that, in the presence of NaCl or LiCl, guanine nucleotides can convert D_2 receptors from high-affinity to low-affinity states. These observations also imply that, depending on the ions and/or nucleotide present, [^{3}H]raclopride can label two states of the D_2 receptor that is coupled via a G protein to adenylate cyclase.

DISCUSSION

The binding of agonists to the D_2 receptor is known to be regulated by Na^+ [24, 25] and guanine nucleotides [10, 26] as well as being sensitive to pertussis toxin [3], suggesting that this receptor is linked to an inhibitory G protein. Antagonist binding is generally unaffected by the addition of Na^+ or Gpp(NH)p to the incubation buffer; however, Na^+ has been found to be an absolute requirement for binding of the benzamide series of D_2 antagonists [12, 27–29]. This effect was found to be specific and only lithium could partially replace sodium [13]. In accordance with these findings, the addition of Na^+ to the incubation medium enhanced the binding of the novel benzamide [^{3}H]raclopride to D_2 receptors in rabbit neostriatum in a concentration-dependent manner. The substitution of Li^+ for Na^+ produced a similar elevation in binding, although lithium was only about 50% as effective as sodium in this respect. In contrast to these two ions, the presence of increasing concentrations of KCl (up to 150 mM) in the incubation buffer did not alter significantly [^{3}H]raclopride binding, although the highest doses (120 and 150 mM) resulted in a slight and negligible elevation in the number of counts (Fig. 1). A similar Na^+/K^+ regulation of [^{3}H]raclopride binding to D_2 receptors in the primate frontal cortex has been reported recently [16], and in both rat and rabbit neostriatum specific [^{3}H] raclopride binding was sodium-dependent [15]. In the present study, the ability of Na^+ and Li^+ to augment [^{3}H]raclopride binding appears to be related primarily to a change in the affinity of the ligand for the D_2 receptor. The saturation binding isotherms for [^{3}H]raclopride

Table 2. Inhibition by dopamine and (+)-butaclamol of specific [^{3}H]raclopride binding to membranes from rabbit neostriatum in buffers with different ionic composition

	N	Dopamine		(+)-Butaclamol	
		IC_{50} (nM)	Hill coefficient (n_H)	IC_{50} (nM)	Hill coefficient (n_H)
NaCl	4	568.6 ± 31.7*†	0.535 ± 0.033	9.93 ± 1.58	1.08 ± 0.03
LiCl	4	352.6 ± 45.4	0.502 ± 0.023	6.29 ± 0.27	1.35 ± 0.06
Sucrose	4	263.6 ± 73.5	0.558 ± 0.053	9.07 ± 1.93	0.90 ± 0.02

The inhibition of raclopride binding was determined by incubating the membrane preparations from rabbit neostriatum with 1 nM [^{3}H]raclopride in the presence of 15–20 concentrations (10^{-12} to 10^{-4} M) of the corresponding unlabeled drug. Values are the means ±SE of four (N) separate experiments, each performed in triplicate. In all cases, nonspecific binding was determined as the counts in the presence of 300 μM (±)- sulpiride. The incubation medium was Tris–Cl buffer (50 mM at pH 7.4) with 5 mM KCl containing either 120 mM NaCl, 120 mM LiCl or 120 mM sucrose. The IC_{50} values were obtained by the iterative analysis INHIBITION [22, 23] and the pseudo-Hill coefficients (n_H) were the slopes of the log $[B/(B_{max} - B)]$ versus the log of the concentration of competing drug.

*,† Significant differences were determined by one-way analysis of variance (ANOVA): *$P < 0.01$ between "NaCl" and "LiCl"; and †$P < 0.01$ between "NaCl" and "Sucrose".

Table 3. Inhibition by dopamine of specific [^{3}H]raclopride binding to membranes from rabbit neostriatum in buffers with different ionic composition

	N	K_H (nM)	K_L (nM)	% R_H	% R_L	K_L/K_H
NaCl	4	97.47 ± 26.41*	4532 ± 872	58.1 ± 3.0	41.9 ± 3.0	46.5
LiCl	4	48.86 ± 5.59	2482 ± 668	56.4 ± 4.5	43.6 ± 4.5	50.8
Sucrose	4	45.45 ± 14.14	1970 ± 619	67.7 ± 7.6	32.3 ± 7.6	43.3

Values are the means ±SE of four (N) separate experiments, each performed in triplicate. In all cases, nonspecific binding was determined as the counts in the presence of 300 μM (±)-sulpiride. The incubation medium was Tris–Cl buffer (50 mM at pH 7.4) with 5 mM KCl containing either 120 mM NaCl, 120 mM LiCl or 120 mM sucrose. The competition parameters for a two-site model were obtained by the analysis LIGAND [19, 20]. K_H = dissociation constant of the high-affinity (D_2^{high}) site in nanomolar; K_L = dissociation constant of the low-affinity (D_2^{low}) site in nanomolar; $\%R_H$ = percentage of D_2^{high} sites; and $\%R_L$ = percentage of D_2^{low} sites. The ratio between the dissociation constants of the low- and high-affinity binding sites is given as K_L/K_H.

* Significant differences were determined by one-way analysis of variance (ANOVA); $P < 0.05$ between "NaCl" and "Sucrose".

Table 4. Inhibition by dopamine of [^{3}H]raclopride binding to membranes from rabbit neostriatum in the presence of different ions with and without Gpp(NH)p

	N		IC_{50} (nM)	Hill coefficient (n_H)
NaCl	3	Control	525 ± 24.8	0.593 ± 0.023
		Gpp(NH)p	2337 ± 342.2*	0.617 ± 0.055
LiCl	3	Control	410 ± 72.2	0.509 ± 0.020
		Gpp(NH)p	1597 ± 260.1*	0.640 ± 0.046
Sucrose	3	Control	290 ± 105.6	0.520 ± 0.075
		Gpp(NH)p	542 ± 304.4	0.509 ± 0.103

The inhibition of raclopride binding was determined by incubating the membrane preparations from rabbit neostriatum with 1 nM [^{3}H]raclopride and increasing concentrations of dopamine (15–20 concentrations; from 10^{-12} to 10^{-4} M) in the presence or absence of the guanine nucleotide 5'-guanylylimidodiphosphate (Gpp(NH)p; 100 μM). Values are the means ±SE of three (N) separate experiments, each performed in triplicate. In all cases, nonspecific binding was determined as the counts in the presence of 300 μM (±)-sulpiride. The incubation medium was Tris–Cl buffer (50 mM at pH 7.4) with 5 mM KCl containing either 120 mM NaCl, 120 mM LiCl or 120 mM sucrose.

The IC_{50} values were obtained by the iterative analysis INHIBITION [22, 23], and the pseudo-Hill coefficients (n_H) were the slopes of the log $[B/(B_{max} - B)]$ versus the log concentration of competing drug.

* Statistical significance between the values obtained in the absence (control) and in the presence of guanine nucleotide was determined by Student's t-test: $P < 0.001$.

Table 5. Effects of Gpp(NH)p on the inhibition by dopamine of specific [^{3}H]raclopride binding to membranes from rabbit neostriatum in buffers of different ionic composition

	N		K_H (nM)	K_L (nM)	$\%R_H$	$\%R_L$	R_H/R_L
NaCl	3	Control	137.1 ± 37.6	5612 ± 1330	62.0 ± 3.8	38.0 ± 3.8	1.63
		Gpp(NH)p	138.9 ± 69.1	5312 ± 826	34.7 ± 1.2*	65.3 ± 1.2*	0.53
LiCl	3	Control	47.3 ± 4.2	1794 ± 603	53.6 ± 8.2	46.4 ± 8.2	1.16
		Gpp(NH)p	48.7 ± 15.3	2377 ± 217	23.9 ± 2.5†	76.1 ± 2.5†	0.31
Sucrose	3	Control	24.9 ± 5.2	1432 ± 630	59.1 ± 8.6	40.9 ± 8.6	1.44
		Gpp(NH)p	50.0 ± 28.4	1176 ± 660	46.2 ± 13.4	53.8 ± 13.4	0.86

Values are the means ±SE of three (N) separate experiments, each performed in triplicate. In all cases, nonspecific binding was determined as the counts in the presence of 300 μM (±)-sulpiride. The incubation medium was Tris–Cl buffer (50 mM at pH 7.4) with 5 mM KCl containing either 120 mM NaCl, 120 mM LiCl or 120 mM sucrose. The competition parameters for a two-site model were obtained by the analysis LIGAND [19, 20]. K_H = dissociation constant of the high-affinity (D_2^{high}) site in nanomolar; K_L = dissociation constant of the low-affinity (D_2^{low}) site in nanomolar; $\%R_H$ = percentage of D_2^{high} sites; and $\%R_L$ = percentage of D_2^{low} sites. The ratio between the proportion of D_2^{high} sites to D_2^{low} sites is given as R_H/R_L.

*,† Statistical significance between the values obtained in the absence (control) and in the presence of guanine nucleotide was determined by Student's t-test; (*) $P < 0.01$ and (†) $P < 0.05$.

performed in buffers containing Na^+, Li^+ or sucrose revealed that both cations significantly increased the affinity of this ligand for the D_2 receptor. Furthermore the affinity change occurring in the presence of Li^+ was two times smaller than that found with Na^+. This observation was consistent with the more modest ability of lithium to enhance [^{3}H]raclopride binding throughout this study. An unexplained finding was that, in contrast to saturation binding curves, the association and dissociation kinetics did not reveal important changes in the apparent K_d between Na^+ and Li^+. However, it could be possible that since saturation experiments are carried out at equilibrium at a time when the rate of association is stable, the faster rate of dissociation of [^{3}H]raclopride found in the presence of Li^+ is reflected as a much lower affinity for the receptor in this buffer. It can also be recalled that cations attenuate the dissociation of [^{3}H]spiperone from the D_2 receptor [30]. This mechanism may also be valid for [^{3}H]raclopride and, in fact, the dissociation of ligand was slowest in the incubation buffer containing Na^+.

As mentioned above, the binding of agonists to the D_2 receptor was sensitive to the addition of Na^+ to the incubation medium. In accordance with these data, the affinity of dopamine to compete with [^{3}H]raclopride binding was decreased in the presence of Na^+ or Li^+. In contrast, the binding of the dopaminergic antagonist (+)-butaclamol was not influenced by the addition of NaCl. The alteration in the affinity of the D_2 receptor for dopamine appeared to be related to a loss of affinity for both the D_2^{high} and D_2^{low} sites and not due to the conversion of the two states of the D_2 receptor as reported for [^{3}H]spiperone [11] or [^{3}H]domperidone [31]. Despite the claim by Niznik and coworkers [29] that the affinity of ADTN for the D_2 receptor labeled by [^{3}H]YM-09151-2 was decreased in the presence of Na^+ due to the reduction in the amount of high-affinity binding, only 5% of D_2^{high} sites were in fact lost. However, Na^+ produced an approximately 8-fold decrease in the affinity of dopamine for the high-affinity state of the receptor. This finding is in accord with the present data and suggests that Na^+ may alter the binding of agonists to the D_2 receptor via a different mechanism for receptors labeled by [^{3}H]spiperone than for those labeled with the benzamide series of antagonists. Alternatively, the discrepancy between these data may be related to the absolute requirements of the benzamide compounds for Na^+. In contrast to spiperone and other classic neuroleptics, the presence of sodium increases the affinity of the benzamide series of antagonists for the D_2 receptor [12, 32] but decreases the affinity of dopaminergic agonists for this site. These paradoxical actions of sodium ions on D_2 receptors labeled with [^{3}H]benzamides may influence the mechanism by which this cation exerts its effect on agonist binding.

Guanine nucleotides regulate the sensitivity of adenylate cyclase to neurotransmitters probably through the coupling of the receptor with cyclase [3–5]. The apparent affinity for agonist binding to dopamine receptors in brain is decreased in the presence of guanine nucleotide and this loss of affinity appears to be due to the conversion of high-affinity sites to low-affinity sites [10, 26]. Interestingly, in the absence of Na^+, guanine nucleotides have little influence on the proportion of the two states of the D_2 receptor, suggesting that cations are required before this conversion can occur [11, 30, 33]. These findings are consistent with the observations of the present study (Tables 4 and 5), which reveal that in the absence of cations 100 μM Gpp(NH)p had little influence on the affinity of dopamine to compete with [^{3}H]raclopride. On the other hand, the addition of either Na^+ or Li^+ to the incubation buffer containing Gpp(NH)p resulted in a significant loss of D_2^{high} sites. The effect of Gpp(NH)p was specific for the agonist (i.e. dopamine) since the presence of this nucleotide did not influence the maximum level of [^{3}H]raclopride binding. The specific influence of guanine nucleotides has been shown for other D_2 ligands [11, 34]. The

loss of D_2^{high} sites documented in this study was not complete, however, and around 20–30% of high-affinity sites remained after treatment with Gpp(NH)p. Whether guanine nucleotides are able to induce total conversion of D_2 receptors is a matter of controversy at present.

The effects of Na^+ and Li^+ on the binding properties of [^{3}H]raclopride and the agonist binding to the D_2 receptor labeled by this ligand were qualitatively similar. These findings indicate that these cations may act at the same site of the ligand–receptor complex. The receptor-mediated inhibition of adenylate cyclase is dependent on the presence of sodium ions which enhance the coupling of the receptor to a G_i protein [35]. The mechanism of this action is unknown, but it has been proposed that these ions may compete with magnesium for a binding site located on the catalytic unit of the G protein [36]. Throughout the present study, binding of [^{3}H]raclopride in medium containing Li^+ was around 50% of that found in the presence of Na^+, suggesting that lithium could only partially replace sodium. This finding is consistent with binding of [^{3}H]sulpiride to rat striatal membranes, where the substitution of Na^+ by Li^+ resulted in a reduction in specific binding to around 55% of maximum [32].

In summary, this study revealed that [^{3}H]raclopride binding to D_2 receptors in rabbit neostriaum was dependent upon the presence of either Na^+ or Li^+, but not K^+, in the incubation buffer. Such an ionic dependency is consistent with that of other benzamide antagonists. The affinity of dopamine to compete for the D_2 receptor labeled by [^{3}H]raclopride was also influenced by the presence of Na^+, Li^+ and the non-hydrolyzable guanine nucleotide Gpp(NH)p. In all circumstances, Li^+ had an effect similar to that of Na^+ on both agonist and antagonist binding, suggesting that this cation was acting at the same site as sodium. The results show both agonist and antagonist affinity changes for the D_2 binding site labeled with this benzamine compound. The findings also show the importance of the incubation buffer used in binding studies [15] as well as the ionic influence on the kinetic parameters. These issues should be taken into account when using [^{3}H]-raclopride for *in vitro* binding experiments as well as for the *in vivo* labeling by positron emission tomography of dopamine receptors.

Acknowledgements—Supported by the Medical Research Council of Canada (Grant MT-6967) and by the Université de Montréal. Personal support was from the Fonds de la recherche en santé du Québec to Dr. T. A. Reader (Chercheur-boursier Senior 2) and to Ms. S. Boulianne (Studentship) and from the Centre de recherche en sciences neurologiques to Dr. Karen M. Dewar (Herbert H. Jasper Fellowship). The technical assistance of Ms. Louise Grondin is gratefully acknowledged.

REFERENCES

1. Kebabian JW and Calne DW, Multiple receptors for dopamine. *Nature* **277**: 93–96, 1979.
2. Onali P, Olianas MC and Gessa GL, Selective blockade of dopamine D-1 receptors by SCH23390 discloses striatal dopamine D-2 receptors mediating the inhibition of adenylate cyclase in rats. *Eur J Pharmacol* **99**: 127–128, 1984.
3. Fujita N, Nakahiro M, Fukuchi I, Saito K and Yoshida H, Effects of pertussis toxin on D_2-dopamine receptors in rat striatum: Evidence for coupling of Ni regulatory protein with D_2-receptor. *Brain Res* **333**: 231–236, 1985.
4. Rodbell M, The role of hormone receptors and GTP-regulatory proteins in membrane transduction. *Nature* **284**: 17–22, 1980.
5. Limbird LE, GTP and Na^+ modulate receptor-adenyl cyclase coupling and receptor-mediated-function. *Am J Physiol* **247**: 59–68, 1984.
6. Seeman P, Grigoriadis D, George SR, Watanabe M and Ulpian C, Functional states of dopamine receptors. In: *Dopaminergic Systems and Their Regulation* (Eds. Woodruff GN, Poat JA and Roberts PJ), pp. 97–109. Macmillan, London, 1986.
7. Seeman P and Grigoriadis D, Dopamine receptors in brain and periphery. *Neurochem Int* **10**: 1–25, 1987.
8. De Lean A, Stadel JM and Lefkowitz RJ, A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase coupled β-adrenergic receptor. *J Biol Chem* **255**: 7108–7117, 1980.
9. Sibley DR, De Lean A and Creese I, Anterior pituitary dopamine receptors. Demonstration of interconvertable high and low affinity states of the D-2 dopamine receptor. *J Biol Chem* **257**: 6351–6361, 1982.
10. Creese I, Usdin T and Snyder SH, Guanine nucleotides distinguish between two dopamine receptors. *Nature* **278**: 577–578, 1979.
11. Grigoriadis D and Seeman P, Complete conversion of brain D_2 dopamine receptors from the high- to the low-affinity state for dopamine agonists, using sodium ions and guanine nucleotide. *J Neurochem* **44**: 1925–1935, 1985.
12. Stefanini E, Marchisio AM, Devoto P, Vernaleone F, Collu R and Spano PF, Sodium-dependent interaction of benzamides with dopamine receptors. *Brain Res* **198**: 229–233, 1980.
13. Freedman SB and Woodruff GN, Effect of cations on [^{3}H]sulpiride binding. *Br J Pharmacol* **74**: 228P, 1981.
14. Köhler C, Hall H, Ögren S-O and Gawell L, Specific *in vitro* and *in vivo* binding of ^{3}H-raclopride. A potent substituted benzamide drug with high affinity for dopamine D-2 receptors in the rat brain. *Biochem Pharmacol* **34**: 2251–2259, 1985.
15. Dewar KM, Montreuil B, Grondin L and Reader TA, Dopamine D_2 receptors labeled with [^{3}H]raclopride in rat and rabbit brains. Equilibrium binding, kinetics, distribution and selectivity. *J Pharmacol Exp Ther* **250**: 696–706, 1989.
16. Lidow MS, Goldman-Rakic PS, Rakic P and Innis RB, Dopamine D_2 receptors in the cerebral cortex: Distribution and pharmacological characterization with [^{3}H]raclopride. *Proc Natl Acad Sci USA* **86**: 6412–6416, 1989.
17. Zahniser NR and Dubocovich ML, Comparison of dopamine receptor sites labeled by [^{3}H]-sulpiride and [^{3}H]-spiperone in striatum. *J Pharmacol Exp Ther* **227**: 592–599, 1983.
18. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–276, 1951.
19. Munson PJ and Rodbard D, LIGAND: A versatile computerized approach for characterization of ligand-binding systems. *Anal Biochem* **107**: 220–239, 1980.
20. McPherson GA, Analysis of radioligand binding experiments: A collection of computer programs for IBM PC. *J Pharmacol Methods* **14**: 213–228, 1985.
21. Scatchard G, The attraction of proteins for small molecules and ions. *Ann NY Acad Sci* **51**: 660–672, 1949.
22. Barlow RB, *Biodata Handling with Microcomputers*, pp. 125–143. Elsevier Science Publishers, Amsterdam, 1983.

23. Parker RB and Waud DR, Pharmacological estimation of drug–receptor dissociation constants. Statistical evaluation. I. Agonists. *J Pharmacol Exp Ther* **177**: 1–12, 1971.
24. Makman MH, Dvorkin B and Klein PN, Sodium ion modulates D_2 receptor characteristics of dopamine agonist and antagonist binding sites in striatum and retina. *Proc Natl Acad Sci USA* **79**: 4212–4216, 1982.
25. Hamblin MW and Creese I, ^{3}H-Dopamine binding to rat striatal D-2 and D-3 sites: Enhancement by magnesium and inhibition by guanine nucleotides and sodium. *Life Sci* **30**: 1587–1595, 1982.
26. Zahniser NR and Molinoff PB, Effect of guanine nucleotides on striatal dopamine receptors. *Nature* **275**: 453–455, 1978.
27. Freedman SB, Poat JA and Woodruff GN, Influence of sodium and sulphydryl groups on [^{3}H]sulpiride binding sites in rat striatal membranes. *J Neurochem* **38**: 1459–1465, 1982.
28. Hall H, Köhler C and Gawell L, Some *in vitro* receptor binding properties of [^{3}H]eticlopride, a novel substituted benzamine, selective for dopamine-D_2 receptors in the rat brain. *Eur J Pharmacol* **111**: 191–199, 1985.
29. Niznik HB, Grigoriadis DE, Pri-Bar I, Buchman O and Seeman P, Dopamine D_2 receptors selectively labeled by a benzamide neuroleptic: [^{3}H]YM-09151-2. *Naunyn Schmiedebergs Arch Pharmacol* **329**: 333–343, 1985.
30. Usdin TB, Creese I and Snyder SH, Regulation by cations of [^{3}H]spiroperidol binding associated with dopamine receptors of rat brain. *J Neurochem* **34**: 669–676, 1980.
31. Grigoriadis D and Seeman P, [^{3}H]Domperidone labels only a single population of receptors which convert from high to low affinity for dopamine in rat brain. *Naunyn Schmiedebergs Arch Pharmacol* **332**: 21–25, 1986.
32. Theodorou AE, Hall MD, Jenner P and Marsden CD, Cation regulation differentiates specific binding of [^{3}H]sulpiride and [^{3}H]spiperone to rat striatal preparations. *J Pharm Pharmacol* **32**: 441–444, 1980.
33. Watanabe M, George SR and Seeman P, Dependence of dopamine receptor conversion from agonist high- to low-affinity state on temperature and sodium ions. *Biochem Pharmacol* **34**: 2459–2463, 1985.
34. Freedman SB, Poat JA and Woodruff GN, Effect of guanine nucleotides on dopaminergic agonist and antagonist affinity for [^{3}H]sulpiride binding sites in rat striatal membrane preparations. *J Neurochem* **37**: 608–612, 1981.
35. Duman RS, Terwilliger RZ, Nestler EJ and Tallman JF, Sodium and potassium regulation of guanine nucleotide-stimulated adenylate cyclase in brain. *Biochem Pharmacol* **38**: 1909–1914, 1989.
36. Newman ME and Belmaker RH, Effects of lithium *in vitro* and *ex vivo* on components of the adenylate cyclase system in membranes from the cerebral cortex of the rat. *Neuropharmacology* **26**: 211–217, 1987.