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DEPENDENCE OF DOPAMINE RECEPTOR CONVERSION FROM AGONIST HIGH- TO LOW-AFFINITY STATE ON TEMPERATURE AND SODIUM IONS

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Abstract—Previous workers found that the anterior pituitary dopamine receptors were inconsistent in converting from their high-affinity state (D_2^{high}) into their low-affinity state (D_2^{low}) for dopamine. We tested, therefore, whether temperature or sodium ion concentration could be factors accounting for such inconsistencies. We found that the proportion of sites which converted depended on the temperature of incubation. No conversion occurred at 4°, despite the presence of guanine nucleotide and sodium ions. At room temperature (20°) guanine nucleotide consistently induced complete conversion in the presence of sodium ions, but gave inconsistent conversion in the absence of sodium ions. At body temperature (37°) guanine nucleotide consistently resulted in complete conversion without requiring sodium ions.

Although it is known that the dopamine D_2 receptor can exist in either a high-affinity state or a low-affinity state for dopamine [1, 2], there have been at least two problems for dopamine receptors in the anterior pituitary gland.

One problem has to do with the fact that the physiological concentration of dopamine reaching the anterior pituitary gland is of the order of 10 nM [3, 4]. However, although *in vitro* functional studies indicate that dopamine inhibits adenylate cyclase [5, 6] and prolactin secretion [7, 8] from anterior pituitary, none of the reported dissociation constants of dopamine at the pituitary dopamine receptor is as low as 10 nM. For example, the lowest values reported from radioligand binding studies are 190 nM [2] and 66 nM [1] for the high-affinity state of the anterior pituitary dopamine receptor, D_2^{high}.

A second problem has been the inconsistency in obtaining a complete conversion of D_2^{high} into D_2^{low}. For example, Sibley *et al.* [2] obtained a complete conversion of D_2^{high} into D_2^{low}, but De Lean *et al.* [1] did not find this conversion to be complete. Since the former study was done at 37° and the latter study at 25°, we decided to examine in the present work whether temperature and other factors (such as sodium ions) were important in controlling the amount of interconversion between D_2^{high} and D_2^{low}. A preliminary abstract of this work has been published [9].

MATERIALS AND METHODS

Membrane preparation. Porcine pituitaries, freshly frozen on dry ice, were purchased from Bocknek Organic Materials (Toronto) and stored at −70° until use. The effects of temperature, NaCl and guanine nucleotide to be reported in this study were identical in fresh and frozen tissue; even after 1 year of storage at −70°, the proportion of D_2 receptors in the high-affinity state was not diminished. On each assay day, pituitaries were thawed on ice, and the neurointermediate lobe and pituitary stalk were dissected away from the anterior lobe. Anterior pituitaries (approximately 200 mg each) were minced finely with scissors, suspended in chilled buffer (pH 7.4 at 0°, containing 50 mM Tris–HCl, 1 mM EDTA acid, 4 mM $MgCl_2$, 1.5 mM $CaCl_2$, 5 mM KCl), disrupted with a Brinkmann Polytron (20 sec at setting 7) and passed through two layers of cheesecloth. The homogenate was then centrifuged at 480 *g* for 10 min at 4°. The supernatant fraction was kept aside, and the pellet was resuspended and centrifuged again similarly in an equal volume of buffer. The combined supernatant fractions were then centrifuged at 48,000 *g* for 30 min at 4°. The resulting pellet was redispersed in buffer containing the same ingredients as above, pH 7.4 at 37°, in a concentration of 200 mg original weight/ml. This membrane suspension was preincubated for 10 min at 37° and then kept chilled until addition to the incubation tubes in 100 μl aliquots to give a final concentration of approximately 100 μg protein/ml of incubation medium.

Binding of [^{3}H]spiperone to homogenates. The binding of [^{3}H]spiperone to dopamine receptors was studied with incubation initiated by the addition of the anterior pituitary membrane suspension to tubes containing buffer (pH 7.4 at 4°, 20° or 37°), as described, with 12.5 μM nialamide, 0.1% ascorbic acid, [^{3}H]spiperone and drug(s) in a final assay volume of 5 ml. The time course of [^{3}H]spiperone equilibration at the various temperatures (4°, 20° and 37°) was obtained, and all subsequent incubations were performed at equilibrium (16 hr at 4°, 75 min at 20°, 35 min at 37°). At the end of the incubation period, bound ^{3}H-ligand was separated from free ^{3}H-ligand by rapid filtration (5–10 sec), through glass

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fibre filter mats using a cell harvester (Skatron Co., Lier, Norway). Following two 10-sec washes (approximately 10 ml), the filters were air-dried, punched into mini-vials, shaken overnight in 4 ml scintillation fluid and monitored in a Packard 460C liquid scintillation spectrometer with an efficiency of 40–50%. All experiments were carried out in triplicate. Specific binding of [^{3}H]spiperone was defined as that inhibited by 1 μM (+)-butaclamol.

Materials. [^{3}H]Spiperone (22–30 Ci/mmole) was purchased from the New England Nuclear Corp. (Boston, MA). 5′-Guanylylimidodiphosphate (Gpp(NH)p) and dopamine hydrochloride were from the Sigma Chemical Co. (St. Louis, MO). (+)-Butaclamol was donated by Ayerst Research Laboratories (Montreal). All other chemicals were analytical grade from commercial sources.

Data analysis. Data were analyzed by a weighted, nonlinear, least squares curve-fitting program (LIGAND, courtesy of Drs. P. Munson and D. Rodbard, National Institutes of Health, Bethesda, MD) [10] with minor modifications, using a Digital Systems DEC-10 computer. Data were fit successively to one population and two populations of binding sites. A two-site model was retained as appropriate only when a statistically significant improvement of the fit to the data was obtained over a one-site model, based on the assumption that deviations of the data from that expected for a one-site bimolecular reaction (law of mass action) resulted from different affinities of the ligand to more than one class of binding site present. Although the ternary complex model proposed by previous researchers [1, 2] is likely the most appropriate for the anterior pituitary D_2 receptor, we have assumed that, under the equilibrium condition of our assay, two subpopulations of binding sites are detectable, which although interconvertible states of a single receptor, may be analyzed using a "two site" model. The parameters $K_D{}^{high}$ and $K_D{}^{low}$ are useful parameters to describe the two populations of binding sites recognized by dopamine and to make statistical comparisons of data using pK_d values [11]. Parameter estimates within the selected model were obtained with iterative fits, with no constraints imposed, such that the best fitted simulated line for a given data set did not differ significantly from the experimental data points. Data were plotted by a Nicolet Zeta-8 plotter using our own program. Statistical evaluations of K_D values (using pK_D) and proportions of receptor in the two states were performed by Student's *t*-test (two-tailed).

RESULTS

Effect of temperature on antagonist [^{3}H]spiperone binding. To analyze [^{3}H]spiperone/agonist competition under varying conditions, it was first essential to document the kinetics of [^{3}H]spiperone binding under those conditions. For this reason, saturation experiments of [^{3}H]spiperone binding were done in the presence and absence of sodium ions and/or guanine nucleotide at various temperatures (4°, 20°, 37°). The results are shown in Fig. 1. Addition of 100 mM NaCl decreased the K_D of [^{3}H]spiperone at any temperature examined. The addition of guanine nucleotide (100 μM Gpp(NH)p), however, had little effect on the K_D value of [^{3}H]spiperone at any temperature. The K_D values for [^{3}H]spiperone were not influenced significantly by the incubation temperature. All Scatchard plots were linear, indicating that a single class of binding sites was labeled by [^{3}H]spiperone, over the wide range of concentrations examined (10 pM to 2 nM). The K_D values under these various conditions in Fig. 1 were subsequently used in the analysis of [^{3}H]spiperone/dopamine competition curves.

Effect of temperature on dopamine K_D *values.* In

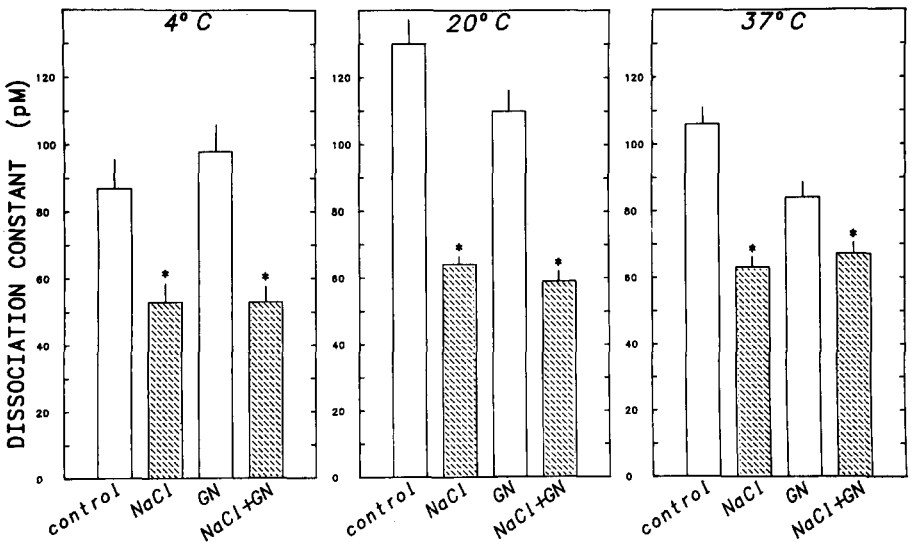

Fig. 1. Dissociation constants (K_D) of [^{3}H]spiperone binding at various temperatures as influenced by 100 mM sodium chloride (NaCl), 100 μM Gpp(NH)p (GN), and the combination (NaCl + GN). Values were obtained from saturation isotherms and Scatchard analyses done in triplicate using [^{3}H]spiperone concentrations of 10–2000 pM. Shown are values ± S.E.M. from N = 3 independent experiments. A significant difference from control at $P < 0.05$ is denoted by an asterisk (*).

control conditions (no sodium ions, no guanyl nucleotides), the analysis of competition curves of [^{3}H]spiperone and dopamine indicated two agonist affinity forms (D_2^{high} and D_2^{low}) at all three different incubation temperatures (4°, 20°, 37°) (Fig. 2). An interesting observation was that the K_D values of the D_2^{high} and D_2^{low} states at 37° were very high as compared with those at 20° and 4° incubations (Fig. 2). In control conditions, there were statistically significant differences ($P < 0.05$) of pK_D values of both sites between 37° and lower incubation temperatures (20° or 4°), while there were no such significant differences of pK_D values between 20° and 4° incubations. The incubation temperature, however, had little effect on the relative proportions of D_2^{high} and D_2^{low} populations.

Temperature and the effects of guanine nucleotide and sodium ions. A representative example of dopamine competition for [^{3}H]spiperone binding at 4° incubation is shown in Fig. 2 (top). Sodium ions (100 mM) did not have a significant effect on the dopamine K_D values or proportions of sites in dopamine/[^{3}H]spiperone competition curves. A 100 μM concentration of Gpp(NH)p shifted the curve to the right, increasing the dopamine K_D values at both the D_2^{high} and D_2^{low} sites, without reducing the proportion of D_2^{high} sites. At 4°, even in the presence of both sodium ions and guanine nucleotide, little conversion of D_2^{high} to D_2^{low} sites occurred.

Typical results of competition experiments of dopamine/[^{3}H]spiperone at 20° are shown in Fig. 2 (middle). At 20° sodium ions alone did not alter the

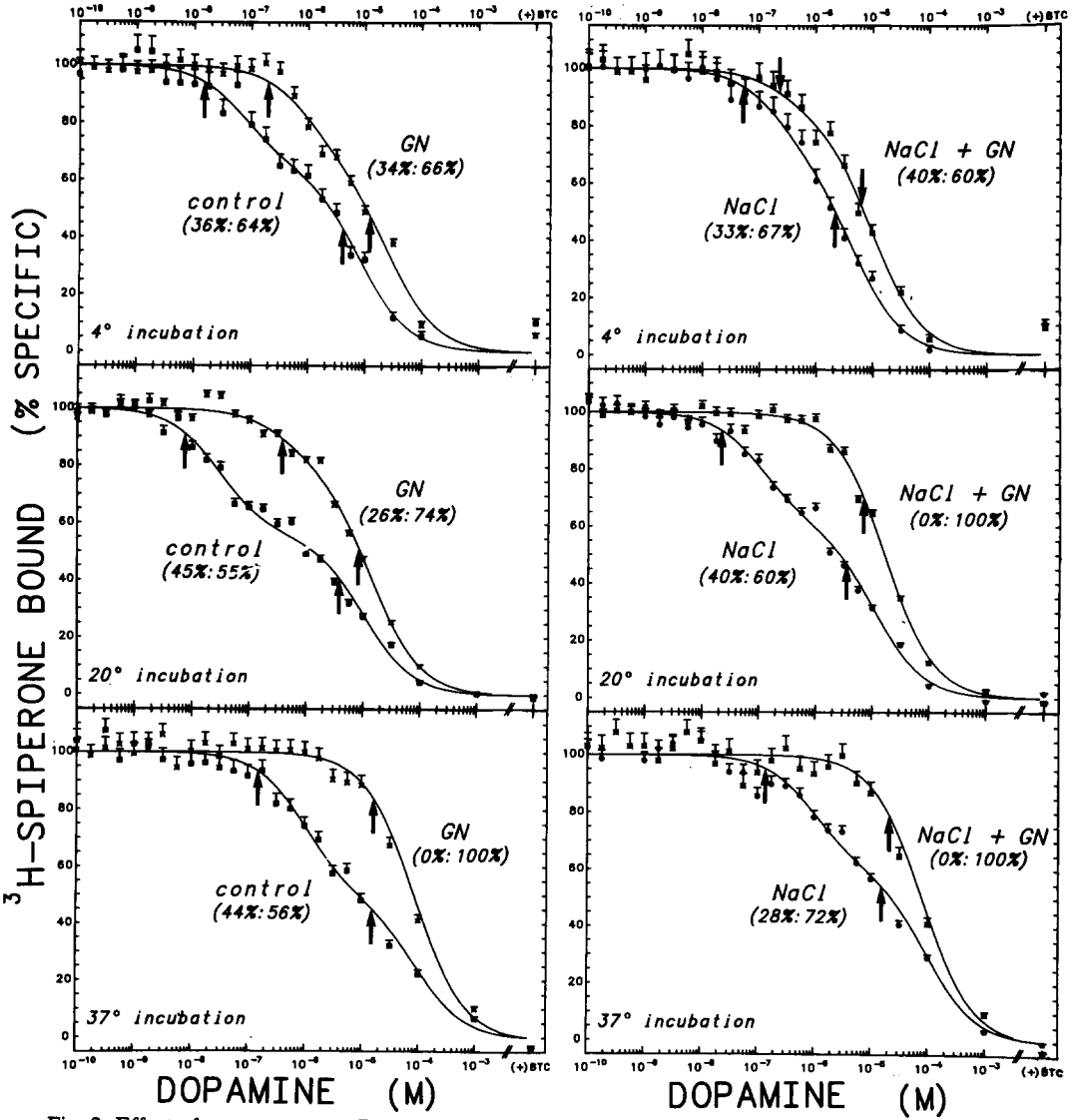

Fig. 2. Effect of temperature on D_2 receptor regulation by NaCl (100 mM) and by guanine nucleotide (100 μM Gpp(NH)p). The dopamine K_D values are denoted by the solid arrows. The proportions of D_2 sites in the high-affinity state and in the low-affinity state are given in parentheses (% D_2^{high}; % D_2^{low}). Each line indicates a representative experiment typical of many for each condition (see Table 1)); each point was in triplicate. Guanine nucleotide alone fully converted the receptors only at 37°. At room temperature (20°), however, the nucleotide required NaCl for full conversion. Very little conversion occurred at 4°, although both NaCl and guanine nucleotide were present. BTC = 1 μM (+)-butaclamol.

Table 1. Effect of 100 mM sodium chloride, guanine nucleotide (100 μM Gpp(NH)p) and temperature on the dissociation constant (K_D) and proportion (%) of D_2 receptors in the agonist high-affinity (D_2^{high}) and low-affinity (D_2^{low}) states, as detected by dopamine competition of [^{3}H]spiperone (200 pM) binding*

Incubation temperature	Receptor state	Control K_D (pK_D)	Control %	NaCl (100 mM) K_D (pK_D)	NaCl (100 mM) %	Gpp(NH)p (100 μM) K_D (pK_D)	Gpp(NH)p (100 μM) %	NaCl + Gpp(NH)p K_D (pK_D)	NaCl + Gpp(NH)p %
4°	D_2^{high}	17 ± 5 nM	40 ± 2	31 ± 10 nM	37 ± 2	357 ± 139 nM	41 ± 13	118 ± 65 nM	29 ± 13
(N = 3)	D_2^{low}	1.6 ± 0.3 μM	60 ± 2	1.1 ± 0.1 μM	63 ± 2	6.4 ± 1.8 μM	59 ± 13	1.9 ± 0.5 μM	71 ± 13
20°	D_2^{high}	13 ± 5 nM	47 ± 4	19 ± 3 nM	40 ± 1	414 ± 76 nM	36 ± 7		0
(N = 3–5)	D_2^{low}	4.3 ± 1.7 μM	53 ± 4	2.0 ± 0.2 μM	60 ± 1	6.7 ± 0.5 μM	64 ± 7	2.9 ± 0.2 μM	100
	D_2^{high}						0		
	D_2^{low}					4.0 ± 0.7 μM	100		
37°	D_2^{high}	180 ± 72 nM	46 ± 2	111 ± 51 nM	31 ± 3		0		0
(N = 3)	D_2^{low}	16.3 ± 5.5 μM	54 ± 2	14.8 ± 3.8 μM	69 ± 3	12.6 ± 4.9 μM	100	17.7 ± 5.1 μM	100

* Values shown are means ± S.E.M. of parameters obtained from the number of separate determinations (N), shown in parentheses. Statistical comparisons were performed using pK_D values [11].

proportion of receptors in the high or low agonist-affinity states or the dopamine K_D values. A 100 μM concentration of Gpp(NH)p had a dramatic effect on the agonist high- and low-affinity states. At 20° incubation, complete conversion of D_2^{high} to the D_2^{low} state could usually, but not always, be obtained by Gpp(NH)p alone. However, in the presence of both sodium ions and guanine nucleotide at 20°, complete conversion of D_2^{high} to D_2^{low} was invariably obtained (Fig. 2, middle; summarized in Table 1).

Typical results at 37° are shown in Fig. 2 (bottom) and summarized in Table 1. At 37° incubation, in control conditions, the proportion of receptors in the agonist high- and low-affinity states was similar to that detected at the other temperatures. Gpp(NH)p alone always converted all the D_2^{high} states to D_2^{low}. Sodium ions alone could only partly convert, the proportions of D_2^{high} decreasing significantly from 46% to 31% ($P < 0.02$). The dopamine K_D values of D_2^{high} and D_2^{low} were not altered by sodium ion at 37°. In the presence of both sodium and Gpp(NH)p, there was only a single population of low-affinity sites remaining at 37° (Fig. 2, bottom). Finally, since 50 mM Na_2SO_4 had the same effect as 100 mM NaCl, this indicated that the converting action of NaCl did not depend on the chloride ions (data not shown).

DISCUSSION

The results indicate that the conversion of D_2^{high} into D_2^{low} in the anterior pituitary is regulated not only by guanine nucleotides, as is well known, but also by temperature and sodium ions, factors not previously reported.

Effect of temperature. The K_D of [^{3}H]spiperone was not influenced by temperature, but 37° incubation temperature increased the K_D of dopamine. This is similar to that reported for β-adrenoceptors, where the agonist K_D values vary with temperature but the antagonist K_D is relatively insensitive to temperature [12, 13]. The same holds for muscarinic receptors [14]. Zahniser and Molinoff [15], however, using rat brain striatum, found that there is an increase in the affinity of the receptor for dopaminergic antagonists with increasing temperature, while the agonist K_D values do not vary with temperature. Hamblin *et al.* [16], on the other hand, reported that increasing temperature increases the agonist K_D values of D_2^{high} and D_2^{low} sites in rat striatal membranes, results similar to our data here in pituitary membranes.

In the present study, we found that the dopamine K_D at D_2^{high} (20°) was 13 ± 12 nM (N = 5), lower than the previously reported values of 190 nM [2] or 66 nM [1]. Thus, a possible reason for the different K_D values for dopamine at D_2^{high} among laboratories could be the incubation temperature, since Sibley *et al.* [2] incubated their tissue at 37°, while De Lean *et al.* [1] incubated at 25°.

Effects of sodium ions. A common feature of receptors which are negatively coupled to adenylate cyclase is their regulation by sodium ions [17, 18]. There have been some reports that D_2 receptors in brain are modulated by sodium ions [19, 20]. Sibley and Creese [21], however, reported that sodium ions

have no effect on D_2 dopamine receptors in anterior pituitary membranes. Sibley and Creese [21], however, used 10 mM NaCl in their experiment, and it is possible that this concentration of sodium ion was not enough to affect the binding. In this present study, however, we found a significant effect of sodium ions on D_2 receptors in the anterior pituitary. The [^{3}H]spiperone K_D was lowered by sodium ions at all temperatures. We have reported this effect elsewhere [22]. The present study also showed that at 20° sodium ions cooperated with guanine nucleotide to induce complete conversion of D_2^{high} to D_2^{low}. At 37° sodium ions alone were able to induce partial conversion of D_2^{high} to D_2^{low}.

Effects of guanine nucleotide. Gpp(NH)p by itself usually fully converted the D_2^{high} to D_2^{low} at 20°, while at 37° Gpp(NH)p always completely converted the sites. We have found previously that Gpp(NH)p alone without sodium ions could not cause conversion in striatal membranes [23]. Although many similarities exist between striatal and pituitary D_2 receptors, some very definite differences have been reported [24]. Different effects of guanine nucleotide on conversion of sites might be one of the differences in regulation of D_2 dopamine receptors between striatum and anterior pituitary. We have also documented a significant decrease in affinities of both D_2^{high} and D_2^{low} at 4° and 20° by Gpp(NH)p in the absence of any change in the proportions of D_2^{high} and D_2^{low}, seen by parallel shifts of the curves to the right (Fig. 2). Such shifts in affinity would not be predicted by current models of receptor–guanine nucleotide interactions such as the ternary complex model [1, 2], and indicate the inadequacy of these models to fully accommodate experimental observations.

The reason for the inconsistent effect of guanine nucleotide at 20° was not clear. However, the incubation temperature of 20° might be very critical for conversion. At 37° full conversion was always observed by guanine nucleotides alone, while at 4° significant conversion did not occur even in the presence of both sodium ions and guanine nucleotide. Thus, the degree of conversion of D_2^{high} to D_2^{low} clearly depended on the incubation temperature. We previously reported that prolonged incubation at 37° by itself could cause full conversion without guanine nucleotides [25].

Leff and Creese [26] found complete conversion in canine striatal tissue at 4° incubation (8 hr). On the other hand, Zahniser and Molinoff [15] reported that at 1° incubation the GTP shift in agonist affinity is abolished in rat striatal membranes. This result appears to be consistent with our results that at 4° there was little conversion by guanine nucleotide.

In summary, we have shown that, *in vitro*, sodium ions and temperature of incubation, in addition to guanine nucleotides, significantly affected the conversion of receptors from the dopamine D_2^{high} state to D_2^{low} in anterior pituitary membranes.

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