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Heat Treatment Mimics Guanosine-5′-Triphosphate Effects on Dopaminergic ^{3}H-Ligand Binding to Bovine Caudate Membranes

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SUMMARY

Exposure of bovine caudate homogenates to 53° rapidly (<4 min) abolishes subsequent specific binding of the agonist ligand [^{3}H]dopamine to D-3 sites but not that of the butyrophenone dopaminergic antagonist [^{3}H]spiroperidol to D-2 sites in bovine caudate membranes. It has been suggested that this represents selective heat inactivation of the binding site for [^{3}H]dopamine. However, the binding of the agonist [^{3}H]apomorphine is decreased with a time course and temperature dependence indistinguishable from that of [^{3}H]dopamine, despite the ability of [^{3}H]apomorphine to label D-2 sites as well as D-3 sites in control membranes. Heat treatment thus appears to mimic the effects on dopaminergic binding of guanine nucleotides, which, rather than causing a reduction in the number of binding sites, radically lowers agonist affinity at both D-2 and D-3 sites while leaving antagonist affinity at these sites unchanged. In addition, heat treatment and maximal guanine nucleotide addition (300 μM GTP) cause identical decreases in the affinities of agonists in the displacement of [^{3}H]spiroperidol, and similar increases in pseudo-Hill coefficient of these displacements. The effects of heat treatment and guanine nucleotides are not additive, suggesting that their effects may involve a common mechanism. Thus, it is strongly suggested that brief exposure to 53° inactivates not the D-3 site itself, but rather a guanine nucleotide binding factor that regulates both D-2 and D-3 agonist affinities and, as in the *beta*-adrenergic system, is essential for formation of high-affinity agonist/receptor complexes.

INTRODUCTION

In the previous paper (1) we demonstrated that dopaminergic D-2 binding sites labeled by [^{3}H]spiroperidol are distinct from D-3 binding sites labeled [^{3}H]dopamine by exploiting the selective irreversible inactivation of D-2 sites by phenoxybenzamine. The agonist ligand [^{3}H]apomorphine was shown to label under these conditions not only D-3 sites, but a major subset of the D-2 sites as well. Recently, Lew and Goldstein (2) described another method to alter irreversibly dopaminergic ^{3}H-ligand binding, apparently in a manner complementary to the effects of phenoxybenzamine. They demonstrated that briefly raising the temperature of striatal homogenates to 53° results in a large decrease in [^{3}H]dopamine specific binding, while leaving [^{3}H]spiroperidol specific binding largely unchanged. This was interpreted as heat-induced selective inactivation of [^{3}H]dopamine binding sites, without any similar denaturation of the sites binding [^{3}H]spiroperidol. Were this the case, it would be expected that [^{3}H]apomorphine binding would be partially resistant to heat inactivation, since 40–60% of [^{3}H]apomorphine binding is to the "heat-stable" receptor. In this paper, we report that this is not the case—[^{3}H]apomorphine binding shows heat sensitivity identical with that of [^{3}H]dopamine. We demonstrate that heat treatment produces changes in dopaminergic ligand binding analogous to those produced for *beta*-adrenergic ligands by disruption of adrenergic receptor interaction with a guanine nucleotide binding protein (3–8). Heat treatment appears to produce its effects on dopaminergic ^{3}H-ligand binding by inactivating such an accessory factor, rather than ligand binding sites per se, suggesting that such a membrane-bound regulatory moiety is also involved in control of dopaminergic agonist binding.

METHODS

General. Tissue preparation, radioligand binding, and ligands used were exactly as previously described (1).

Heat treatment of homogenates. An initial washed membrane pellet was obtained from frozen bovine caudate as described in ref. 1. For standard heat treatment

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studies the washed pellet was resuspended at 100 mg of tissue (wet weight) per milliliter in ice-cold 50 mM Tris-HCl buffer (pH 8.0 at 25°) containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 μM pargyline. This suspension was preincubated for 10 min at 37° after which heat treatment was initiated by rapidly mixing 2-ml aliquots with 10 volumes of the same buffer preheated to 54°. This resulted in a thermally homogenous solution at 53° ± 1°. Control aliquots were added to 10 volumes of buffer at 0°. After the specified treatment time at 53°, the treatment was stopped by rapid mixing with 15 ml of the same buffer at 0°. This lowered the suspension temperature immediately to 31°. Separate experiments demonstrated that the presence of pargyline, as well as exposure of the membrane suspension to temperatures of 37°, as in the preincubation, or 31°, as after stopping heat treatment, had no measurable effect on ^{3}H-ligand binding. After addition of cold buffer, the cooled suspension was immediately centrifuged (10 min at 50,000 × *g* in a Sorvall RC-5B refrigerated centrifuge) and the pellet was resuspended in assay buffer for radioligand binding.

For initial studies on the effect of buffer composition on heat inactivation, the initial washed pellet was suspended in various 50 mM Tris-HCl buffers with additions as specified. Tubes containing 10-ml aliquots (initially at 0°) were immersed in a 53° water bath for 10 min resulting in a homogenate temperature of 44 ± 3° at 5 min and 53 ± 1° at 10 min. After heat exposure, the tubes were plunged into ice, centrifuged, and assayed for radioligand binding, as above.

Materials. The sources of the drugs were as reported in ref. 1. The sources of nucleotides were as follows: guanosine 5′-diphosphate, sodium salt (GDP) and guanosine 5′-triphosphate, sodium salt (GTP), P-L Biochemicals, Inc. (Milwaukee, Wisc.); adenosine 5′-diphosphate, sodium salt (ADP), Sigma Chemical Company (St. Louis, Mo.).

RESULTS

Heat inactivation of [^{3}H]dopamine and [^{3}H]spiroperidol binding: buffer composition and temperature dependence. As reported previously, under certain conditions heat treatment of homogenates caused a selective decrease in the binding of dopaminergic ^{3}H-agonists (2). Incubation of homogenates in a 53° water bath for 10 min caused a marked reduction in subsequent [^{3}H]dopamine specific binding under all conditions studied. However, we found that variations in pH or the addition of ions, known to stabilize [^{3}H]spiroperidol binding (9), or of ascorbic acid, an antioxidant commonly used in binding experiments, had a substantial effect on the degree of reduction in [^{3}H]spiroperidol binding and thus the selectivity of heat treatment. In the presence of 0.1% ascorbic acid, susceptibility of [^{3}H]spiroperidol binding to heat inactivation was increased, decreasing the selectivity of the treatment between binding sites for the two ligands. However, inclusion of a physiological ion mix (120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$) increased the selectivity of the treatment, both enhancing the stability of [^{3}H]spiroperidol binding and decreasing that of [^{3}H]dopamine binding. [^{3}H]Spiroperidol binding susceptiblity was also less at pH 8.0 than at lower pH. Subsequent heat inactivation was therefore always performed in pH 8.0 buffer in the presence of ions, without ascorbic acid to maximize selectivity.

Exposure of homogenates to 53° for 4 min was more selective in decreasing ^{3}H-agonist versus ^{3}H-antagonist binding than comparable exposure to temperatures of 45° or 60° (Fig. 1). Exposure to 45° was less effective in elimination of [^{3}H]dopamine and [^{3}H]apomorphine binding, whereas exposure to 60° decreased [^{3}H]spiroperidol binding as well as ^{3}H-agonist binding. In contrast to the selectivity observed for phenoxybenzamine inactivation (1), there was no significant difference between the effects on the two ^{3}H-agonist ligands at any of the three treatment temperatures.

Time course of heat inactivation at 53°. Exposure of the caudate homogenates caused a rapid decrease in specific binding of the agonist ligands [^{3}H]apomorphine and [^{3}H]dopamine with more than one-half of specific binding eliminated within 30 sec (Fig. 2). The kinetics of inactivation were approximately first-order. The binding of [^{3}H]spiroperidol was affected to a much lesser degree. Unlike treatment with phenoxybenzamine (1), heat treatment affected the binding of [^{3}H]apomorphine and [^{3}H]dopamine equally.

Saturation of [^{3}H]apomorphine binding after homogenate heat treatment. Heat treatment-induced reduction in [^{3}H]apomorphine specific binding was mediated by a reduction in apparent receptor B_{max}, with little change in receptor affinity (Fig. 3). Lew and Goldstein (2) previously reported that the decrease in [^{3}H]dopamine binding was also due to a decrease in receptor number, without change in affinity, a result we have confirmed. Thus in this respect, as well as in time course, the decrease in [^{3}H]apomorphine and [^{3}H]dopamine high-affinity bind-

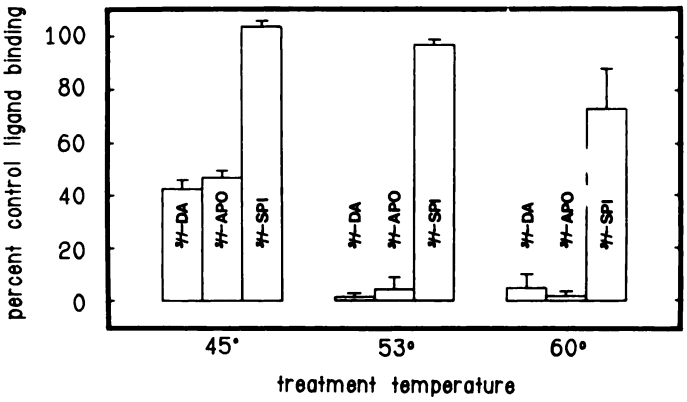

FIG. 1. *Heat inactivation at various temperatures*

Small volumes of tissue homogenate were preincubated at 37° for 10 min and then rapidly brought to 45°, 53°, or 60° by mixing with preheated buffer. After 4 min, the heat treatments were stopped with the addition of a large volume of 0° buffer. Homogenates were centrifuged and then resuspended in assay buffer for [^{3}H]spiroperidol (*^{3}H-SPI*) (0.5 nM), [^{3}H]apomorphine (*^{3}H-APO*) (0.8 nM), and [^{3}H]dopamine (*^{3}H-DA*) (3 nM) binding determinations. Results shown represent means of two to six experiments ± standard error of the mean. Control binding was that of homogenates preincubated and exposed to 37° for a total of 14 min.

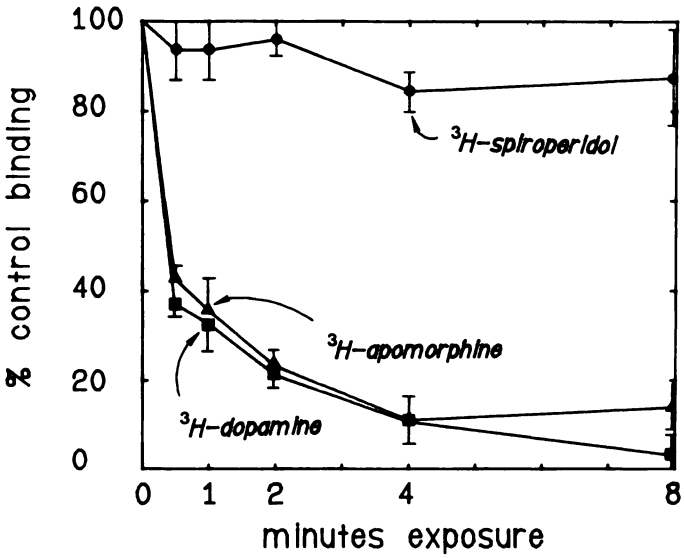

FIG. 2. *Time course of heat inactivation of dopaminergic ^{3}H-ligand binding*

Small volumes of tissue homogenate were rapidly brought to 53° by mixing with preheated buffer. After the times shown, the exposure was stopped by the addition of a large volume of 0° buffer. Results are expressed as means ± standard error of the mean (n = 2–7) percentage of specific binding to unheated homogenates for 0.5 nM [^{3}H]spiroperidol, 0.8 nM [^{3}H]apomorphine, and 3 nM [^{3}H]dopamine.

ing sites appeared identical, despite their difference in phenoxybenzamine sensitivity. A decrease in B_{max} without change in K_D was also observed for [^{3}H]dopamine and [^{3}H]apomorphine binding in homogenates exposed to temperatures of 45° (data not shown).

Displacement of [^{3}H]spiroperidol binding by agonists and the effect of guanine nucleotides after heat treatment. We confirmed the observation (2) that heat treatment, in addition to causing an apparent reduction in the number of high-affinity ^{3}H-agonist binding (D-3) sites, also had a second effect in causing a reduction in potency

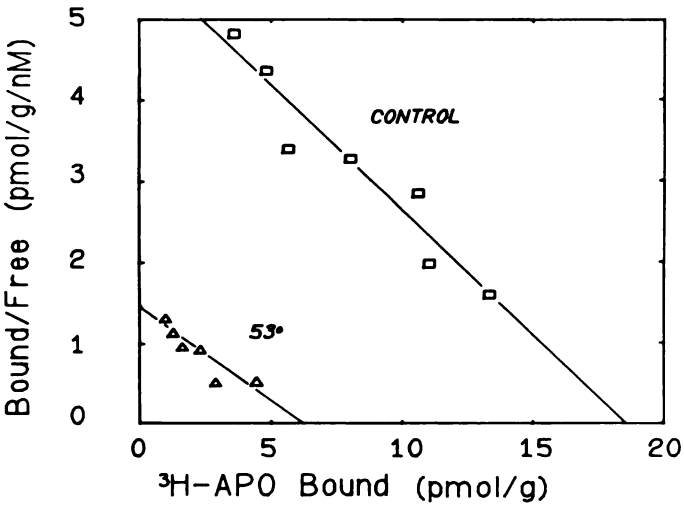

FIG. 3. *Scatchard plot of [^{3}H]apomorphine saturation after homogenate exposure to 53° for 1 min*

After exposure, homogenates were resuspended in assay buffer for binding. [^{3}H]Apomorphine concentrations were 0.5–10 nM. Results represent a single typical experiment, which was repeated twice.

of unlabeled agonists in the displacement of [^{3}H]spiroperidol from D-2 sites. The IC_{50} values for [^{3}H]spiroperidol displacement by dopamine and apomorphine were 14.6-fold (SEM 3.4) and 10.5-fold (SEM 1.5) lower, respectively, after exposure of homogenates to 53° for 4 min. Similar results were obtained with homogenates treated at 45° rather than 53°. The presence of micromolar concentrations of GTP, GDP, or the stable GTP analogue guanylyl-5′-imidodiphosphate is also known to cause a similar decrease in agonist potency at D-2 sites (10–13). We found that heat treatment completely abolished this guanine nucleotide sensitivity of [^{3}H]spiroperidol displacement by both dopamine (Fig. 4) and apomorphine (not shown). Guanine nucleotides also cause an increase in the pseudo-Hill coefficient in agonist displacement of [^{3}H]spiroperidol from D-2 sites (11, 13). Again, heat treatment caused a similar increase in the Hill coefficient observable by the steepening of the displacement curve, as seen with guanine nucleotides (Fig. 4). The pseudo-Hill coefficient for dopamine displacement of [^{3}H]spiroperidol was increased from 0.40 ± 0.05 for control homogenates to 0.78 ± 0.01 in those exposed to 53° for 4 min.

Thus the binding alterations seen with guanine nucleotides and heat treatment were similar in decreasing ^{3}H-agonist but not ^{3}H-butyrophenone binding to D-2 sites, in decreasing agonist potency in ^{3}H-butyrophenone displacement from D-2 sites, and in increasing the pseudo-Hill coefficient of these displacements.

Protection of ^{3}H-agonist binding sites from heat inactivation by dopamine and guanine nucleotides. The presence of 1 μM unlabeled dopamine or 10 μM GDP or

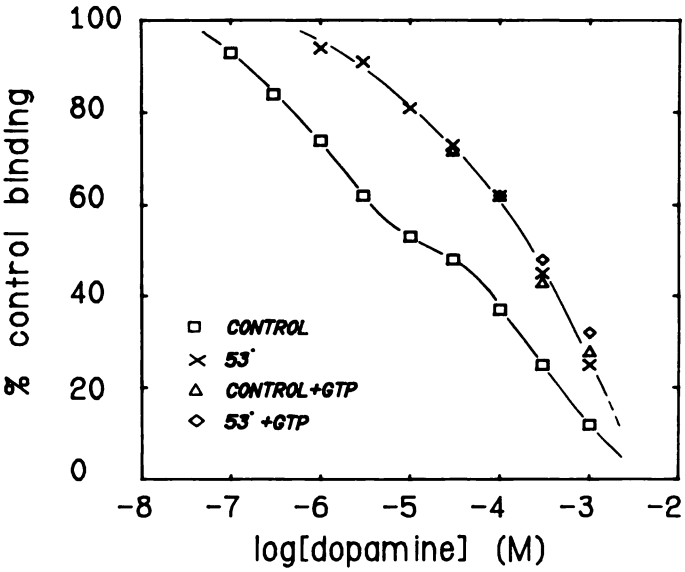

FIG. 4. *Displacement by dopamine of [^{3}H]spiroperidol binding in heat-treated caudate homogenates with or without added GTP.*

Aliquots of homogenate and 0.5 nM [^{3}H]spiroperidol, with or without 1 μM (+)-butaclamol blank, were incubated with various concentrations of unlabeled displacing dopamine, with or without 300 μM GTP. The 53° homogenates were treated at 53° for 4 min. *Control* homogenates were treated identically except for heat treatment. Each point represents the mean of two independent determinations with SEM <15%. Similar results were obtained when 100 μM guanylyl-5′-imidodiphosphate was used rather than 300 μM GTP.

TABLE 1

Protection by dopamine and nucleotides of ^{3}H-agonist binding against heat inactivation

Aliquots of tissue homogenate, preincubated, with or without protecting agent, were rapidly brought to 53° by mixing with preheated buffer, to which the same protecting agent had been added seconds before the tissue. Heat exposure was stopped after 30 sec by addition of 0° buffer. Specific binding of [^{3}H]dopamine (3 nM) and [^{3}H]apomorphine (0.8 nM) was determined after thorough washing to remove protecting agent. Control tissue, representing 100% specific binding, was treated identically except that 0° stop buffer was added to preheated buffer before addition of tissue. Addition of protecting agents had no significant effect on control specific binding. Results are expressed as percentage protection, where control homogenate binding defines 100% protection, and binding for homogenates exposed without protecting agent defines 0% protection. Specific binding in the absence of protecting agents was reduced to 49.4 ± 2.6% [^{3}H]dopamine and 42.0 ± 3.7% [^{3}H]apomorphine of that for control tissue. Each value is the mean ± standard error of the mean for n determinations (in parentheses).

Agonist	% protection with protecting agent				
	10 μM GDP	10 μM GTP	1 μM Dopamine	10 μM GTP + 1 μM Dopamine	10 μM ADP
[^{3}H]Dopamine	35.5 ± 8.6[a] (6)	27.9 ± 5.2[a] (3)	49.6 ± 9.2[a] (9)	59.7 ± 2.1[a] (2)	−5 ± 0 (2)
[^{3}H]Apomorphine	55.8 ± 9.9[a] (5)	38.9 ± 7.8[a] (3)	46.0 ± 7.6[a] (7)	64.2 ± 7.2[a] (2)	

[a] Different from zero protection ($p < 0.05$).

GTP (which have similar effects on dopaminergic binding) during exposure of the tissue homogenate to 53° for 30 sec significantly decreased the heat-mediated inhibition of both [^{3}H]dopamine and [^{3}H]apomorphine specific binding (Table 1). Inclusion of both 1 μM dopamine and 10 μM GTP protected [^{3}H]dopamine and [^{3}H]apomorphine specific binding to a slightly greater extent than either agent alone, although this increase relative to the protection afforded by the agents individually was not statistically significant. ADP at a 10 μM concentration was without a protective effect. Protection afforded by antagonist occupancy was difficult to examine because exposure of membranes to saturating concentrations of the antagonist (+)-butaclamol (1 μM) without heat treatment reduced specific binding to 60% of control values, even after five washes with 25° or 37° buffer. As the decreases in ^{3}H-agonist binding observed with heat treatment and (+)-butaclamol appeared to be additive, however, there was no indication that antagonist occupancy protected against the effects of heat treatment, in contrast to the protection observed for dopamine and guanine nucleotides.

DISCUSSION

Preincubation of homogenates at 53° irreversibly eliminates [^{3}H]dopamine specific binding but not that of [^{3}H]spiroperidol (2). This has been explained as a heat-induced loss of high affinity [^{3}H]dopamine binding (D-3) sites. However, we have presented indirect evidence here suggesting that the effects of heat treatment—the decrease in high affinity ^{3}H-agonist binding to both D-2 and D-3 sites, the decreased potency of agonists in competing for the separate [^{3}H]spiroperidol binding to D-2 sites, and an increased pseudo-Hill slope in those displacements—may be mediated by a different, single event: the loss of an N protein.[3]

[^{3}H]Apomorphine labels nearly equal numbers of the separate D-2 and D-3 sites labeled with high affinity, respectively, by [^{3}H]spiroperidol and [^{3}H]dopamine (1). It would be expected then that the heat sensitivity of binding of [^{3}H]apomorphine would be intermediate to the sensitivity of [^{3}H]spiroperidol and [^{3}H]dopamine binding, if, as is the case for phenoxybenzamine sensitivity (1), this reduction was mediated via direct binding site alteration. This was not the case: the temperature dependence for the decrease in binding of the two ^{3}H-agonist ligands was identical and proceeded with indistinguishable time courses. Thus, heat treatment eliminated ^{3}H-agonist binding, whether to D-2 sites labeled by [^{3}H]apomorphine or to D-3 sites labeled by both [^{3}H]apomorphine and [^{3}H]dopamine. At the same time, this treatment left [^{3}H]spiroperidol binding to D-2 sites unaffected. In addition, GDP, GTP, and dopamine had protective effects against heat-induced inhibition of binding which was similar for [^{3}H]apomorphine and [^{3}H]dopamine despite the ability of these two ligands to label different populations of dopaminergic binding sites under these conditions. Heat inactivation mimicked the actions of guanine nucleotides in causing a decrease in agonist, but not antagonist, specific binding (10–12), a decrease in potency of agonists in the displacement of [^{3}H]spiroperidol (12, 13), and an increase in the pseudo-Hill coefficient in these displacements to a value closer to unity (11, 12).

Guanine nucleotides are thought to regulate receptors coupled with adenylate cyclase through an intermediary accessory protein variously known as "N" (14), "G/F" (15), "G" (16, 17) or "guanine nucleotide regulatory protein" (18). This protein possesses the binding site for guanine nucleotides through which they exert their regulatory actions on both adenylate cyclase activity and receptor agonist affinity (14, 15). A similar regulation of agonist binding by guanine nucleotides is seen in the binding of ligands to numerous other receptor types both unlinked and inhibitory cyclase (14). This has led to the suggestion that guanine nucleotides exert their effects on all receptors so regulated, whether stimulatory, inhibitory, or unrelated to adenylate cyclase, through such membrane-bound guanine nucleotide binding factors. Thus, although neither the D-2 sites labeled by [^{3}H]spiroperidol nor the D-3 sites labeled by [^{3}H]dopamine appear to be the receptor mediating the dopamine stimulation of adenylate cyclase, this mechanism may be applicable at D-2 and D-3 sites as well. Indeed, recent evidence suggests that D-2 receptors in the anterior

[3]The abbreviation used is: N protein, guanine nucleotide-binding regulatory protein.

pituitary are negatively linked to an adenylate cyclase (19–22), which, with our data, is consistent with the hypothesis (14) that receptors with different effects on adenylate cyclase may share common mechanisms of guanine nucleotide regulation.

For the *beta*-adrenergic receptor, the best-characterized guanine nucleotide-regulated system to date, the ability of agonists to displace labeled antagonists with high affinity is dependent on the physical coupling of the receptor to "N," a coupling promoted by agonist receptor occupancy (16). This association, and thus high-affinity ^{3}H-agonist binding, can be prevented by the addition of guanine nucleotides. This same effect, a decrease in N association with receptor and a concomitant decrease in agonist but not antagonist affinity, is seen with a number of manipulations selectively interfering with N function. Such results are seen in S49 lymphoma cells selected for defective N protein (termed *UNC*) (3) or deficient in N (termed *cyc*$^-$) (4), or where N activity is eliminated developmentally, as in maturing rat reticulocytes (5) or chemically with *N*-ethylmaleimide (6–8). The right-shifts and increased Hill slopes in agonist/labeled antagonist displacements produced by all four of these manipulations are identical with those seen with maximal guanine nucleotide addition, just as was the case for the effects of heat treatment on D-2 binding. We therefore suggest that dopaminergic-ligand binding alterations seen in agonist displacement of [^{3}H]spiroperidol from D-2 sites after heat treatment may reflect permanent conformational or other changes in a guanine nucleotide-binding regulatory factor. This elimination of a necessary N protein would also result in the observed absence of high-affinity ^{3}H-agonist binding to the D-3 sites after heat treatment, if indeed, as for the adrenergic system, a receptor/N protein complex was essential for high-affinity agonist binding to D-2 sites (for [^{3}H]apomorphine) and D-3 sites ([^{3}H]apomorphine and [^{3}H]dopamine). No D-3-selective antagonist radioligand is currently available to assess changes in antagonist binding and agonist/labeled antagonist competition at this site. We have also found that the binding of [^{3}H]flupentixol, an antagonist shown to label in part the adenylate cyclase-linked (D-1) dopamine receptor in brain (23, 24), is also relatively heat-stable. The availability of a better D-1-specific ligand may eventually allow assessment of agonist affinity changes with heat treatment at this site as well.

Indeed, heat sensitivity of agonist, but not antagonist, high-affinity binding may be a common factor of many receptor systems. Recently, a 5-min 50° treatment of mouse brain stem homogenates was found to affect muscarinic ligand affinities in just this fashion (25). Also consistent with this is the protective effect exerted by dopamine and guanine nucleotides (but not the antagonist (+)-butaclamol), the binding of which might affect coupling between receptor and regulatory protein, and thus might slow any denaturing conformational change.

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