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MOLECULAR PHARMACOLOGY, 33:155-162

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Guanyl Nucleotide Interactions with Dopaminergic Binding Sites Labeled by [^{3}H]Spiroperidol in Human Caudate and Putamen: Guanyl Nucleotides Enhance Ascorbate-Induced Lipid Peroxidation and Cause an Apparent Loss of High Affinity Binding Sites

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Received February 24, 1987; Accepted November 3, 1987

SUMMARY

The human caudate and putamen contain two high affinity binding sites for [^{3}H]spiroperidol. Both of these affinity states exhibit dopaminergic selectivity. Ascorbic acid, at 0.1 mM, induces a slow loss of the low affinity component of [^{3}H]spiroperidol binding in these tissues. The addition of guanyl nucleotides to the ascorbate produces a more rapid loss of [^{3}H]spiroperidol binding which includes a loss of the highest affinity state for [^{3}H]spiroperidol. Ascorbate induces lipid peroxidation in human caudate and putamen, an effect that is further enhanced by guanyl and inosine nucleotides. In the absence of ascorbate, guanyl nucleotides have no effect on [^{3}H]spiroperidol binding but do decrease the affinity of dopamine at each affinity state >60-fold. In the absence of ascorbate, guanyl nucleotides apparently decrease agonist affinity at human brain dopamine$_2$-binding sites without causing an interconversion of agonist affinity states.

Guanyl nucleotides are known to reduce the affinity of agonists for [^{3}H]spiroperidol-labeled dopamine receptors in lower mammalian striatum and pituitary (1–5). In these tissues, dopamine binds at two components of [^{3}H]spiroperidol-labeled binding sites, each of which has different affinities for the agonist. Data from several studies (2–5) suggest that the reduction of agonist affinity produced by guanyl nucleotides is due to an interconversion of the higher affinity state for agonist into the lower affinity state (2–5). However, the data from at least one study were consistent with a reduction in the affinity for agonist at each affinity state with no interconversion of agonist affinity states (6). Importantly, ascorbate was present at concentrations ranging from 0.1 to 5.7 mM in the former studies (1–5), whereas ascorbate was not included in the latter study (6). In the presence of ascorbate, guanyl nucleotides cause a loss of the highest affinity state for [^{3}H]spiroperidol binding in rat striatum (7). Furthermore, ascorbate induces lipid peroxidation, as measured by the production of TBARs in rat striatum, and in some cases this lipid peroxidation is coincidental with a loss of specific [^{3}H]spiroperidol binding (8). We hypothesized that in the presence of ascorbate, guanyl nucleotides cause an apparent interconversion of affinity states for agonist at the dopamine$_2$ receptor, but in the absence of ascorbate, guanyl nucleotides cause a reduction in agonist affinity for each of the affinity states. We further postulated that the mechanism by which guanyl nucleotides caused an apparent interconversion of affinity states in the presence of ascorbate might be related to lipid peroxidation. The following studies were performed to test these hypotheses.

Materials and Methods

Tissue retrieval and preparation. Postmortem human caudate and putamen were obtained at the time of autopsy according to strict guidelines of the University Hospitals of Cleveland and Cleveland Metropolitan General Hospital from normal specimens, defined as those having cardiovascular or sudden accidental death, absence of neurological or psychiatric disease, resuscitative attempt, and prior medication as determined by medical record and by autopsy results. The average age of the postmortem specimens used was 41.7 ± 13.7 years (range: 18–74 years) with a postmortem interval of 16.2 ± 10.5

This work was supported in part by National Institutes of Health Grant RO1AG04460 to A. C. A. and Grants R23DK35469 and RO1DK31505 to B. R. B.

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ABBREVIATIONS: TBAR, thiobarbituric acid-positive reactant; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TCA, trichloroacetic acid; TBA, 2-thiobarbituric acid; MDA, malondialdehyde; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol.

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hr (range: 1–72 hr). The caudate and putamen were dissected bilaterally, placed in ice-cold 20 mM Na/Hepes (pH 7.4 at 4°) plus 2 mM $MgSO_4$, minced on ice, and quickly frozen in a dry ice and acetone bath prior to storage at −70°.

At the time of preparation, the tissue was thawed at 37° before suspension in 9 volumes of buffer (v/v) and homogenization in a hand-held glass and Teflon homogenizer (10 strokes). The homogenate was diluted to a final 50-fold dilution in the same buffer and centrifuged at 39,000 × *g* for 20 min. The rinsed pellets were resuspended in fresh buffer to the 50-fold dilution, and the entire washing procedure was repeated a total of four times. The final concentrated pellets were stored at −70° following quick freezing in the same buffer (pH 7.4 at 37°). Protein concentrations were determined by the method of Lowry *et al.* (9) with bovine serum albumin in the Hepes/$MgSO_4$ buffer as the standard.

Materials. [^{3}H]Spiroperidol at a specific activity of 50–100 Ci/mmol was obtained from Amersham Corp. (Arlington Heights, IL), and thin layer chromatography in multiple solvent systems was used to determine that radioligand purity was maintained at >98% during continued use. GTP was purchased from Boerhinger Mannheim (Indianapolis, IN) and Sigma Chemical Co. (St. Louis, MO). Haloperidol was generously donated by Jannsen Pharmaceuticals, Inc. (Brunswick, NJ), and desferrioxamine was a gift from CIBA-Geigy (Summit, NJ). Trolox was purchased from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were purchased from Sigma.

Binding assay. [^{3}H]Spiroperidol was dried under N_2 and resuspended in buffer, as previously described (10), for final concentrations of ingredients in a 1.0-ml assay volume as follows: 0.2 nM [^{3}H]spiroperidol, 34.4 mM Na/Hepes (pH 7.4 at 37°), and 0.2 ± 0.05 mg of protein/ml of assay. $MgSO_4$ (3.4 mM) was present under some conditions, as stated, but was omitted in the presence of nucleotides. Other additions were as stated. The reaction was allowed to incubate at 37° for the times stated prior to termination by rapid filtration over Schleicher & Schuell (Keene, NH) glass fiber filters (no. 30). The filters were washed with 15 ml of 5 mM Na/Hepes (pH 7.4 at 4°) plus 2 mM $MgSO_4$ and dried under full vacuum. The entire filtration and washing procedure took <6 sec. Filters were placed in 4 ml of Beckman Econosolve and radioactivity was determined by liquid scintillation spectrophotometry at an efficiency of 30%. Sufficient counts were accumulated to effect a counting error of <5%. Total binding was determined in the absence of additions. Nonspecific binding was determined in the presence of 10 μM haloperidol, and specific binding was taken as the difference in [^{3}H]spiroperidol bound between the two. To ensure sufficient counts at very low radioligand concentrations (<0.1 nM), assay volumes and assay constitutent ingredient volumes were doubled or tripled. The volume of the assay has no effect on the subsequent binding data (not shown).

TBAR assay. The TBAR assay was as described by Kohn and Liversedge (11) as modified by Recknagel *et al.* (12). The standard curve was prepared by placing 0–10 nmol of 1,1,3,3,tetraethoxypropane in the following ingredients (final concentrations): 2.2% TCA, 0.06 N HCl, 0.052 mM TBA for a final volume of 4.5 ml. The test tubes were incubated at 100° for 10 min and cooled on ice; then, absorbance was determined at 532 nm on a visible spectrophotometer. Samples (500 μl) were prepared as for a binding assay (0.2 mg of protein/ml assay in 34.4 mM Na/Hepes and other additions as stated). The samples were incubated for the times stated at 37° and were then diluted in TCA for a final concentration of 5% TCA. This suspension was centrifuged at 1700 × *g* for 10 min. The supernatant was diluted in TBA and HCl for final concentrations as per the standard curve. Results are presented as nmol of MDA/mg of protein. For some studies (see below), absorbance was also determined at 450 nm. In this case, the samples were boiled for 20 min, and absorbance was determined in the hot samples, as the pigment producing absorbance at 450 nm is unstable (see below).

Data analyses. The results are reported as the mean ± standard deviation except those from the computer-assisted analyses, which are reported as the mean ± standard error. Differences between means were determined by Student's *t* test. All experiments were performed in duplicate and there was <7% coefficient of variation between duplicates. Equilibrium rate constants and B_{max} were determined from computer-assisted analyses of the saturation data utilizing LIGAND (13). Saturation experiments performed under a single condition were co-analyzed, first with nonspecific binding constrained to that observed, and then with nonspecific binding a free parameter as recommended (13). A minimum of 44 points obtained in separate experiments were analyzed for each condition. When a multi-site fit is reported, it was the preferred fit as determined by *F* test at $p < 0.05$. In the case of dose response analyses (a minimum of two studies employing at least 35 concentrations of competitor), the kinetic parameters for the radioligand were fixed at the values determined in the saturation analyses and the kinetic parameters for the competitor were free. A fit of the data in which the affinities of the competitor were considered to be unequal at both [^{3}H]spiroperidol affinity states was compared to a fit in which the affinities of competitor were considered equal at both affinity states and to a fit in which the affinity of competitor was considered neglible for one of the [^{3}H]spiroperidol affinity states. Analyses of a three-site fit, assuming [^{3}H]spiroperidol labeled a third site with K_A equal to the lower affinity site, was also attempted but in all cases were less probable than the preferred two-site fit. For both saturation and dose response analyses, initial analyses were performed in which the correction factors between experiments were considered free parameters; then, these were held constant at their best approximation from the most complex model that could be fit. The results of this final analysis are those reported.

Each repetition of an experiment was performed on a different postmortem specimen. This was done to avoid basing conclusions on the study of one human specimen. Unfortunately, however, relatively larger standard deviations are observed than might be expected in studies of a homogeneous animal sample due to the inherent heterogeneity of human studies.

Results

Both [^{3}H]spiroperidol binding components in human caudate and putamen exhibit dopaminergic selectivity. It had not been clear whether both [^{3}H]spiroperidol binding components in human caudate and putamen were dopaminergic (14, 15). Therefore, dose response studies (Table 1) were performed in the absence of ascorbate using a minimum of 35 concentrations of domperidone, (±)-sulpiride, ketanserin, dopamine, and serotonin and a 60-min incubation. Dopamine had a higher affinity at each affinity state than serotonin (Table 1). To determine whether the dopaminergic selectivity at either [^{3}H]spiroperidol binding component was lost in the presence of ascorbate, the dopamine and serotonin studies were repeated with a 30-min incubation in the presence of 0.1 mM ascorbate. The addition of ascorbate did not alter the fact that dopamine was still the agonist with the highest affinity at each [^{3}H] spiroperidol binding component (Table 1). The different incubation times were chosen to assure performance of the studies under each condition at steady state as determined from the rate kinetic studies (see below).

The effects of ascorbate and guanyl nucleotides on [^{3}H]spiroperidol binding. To determine whether there were any potential interactive effects of ascorbate and guanyl nucleotides on [^{3}H]spiroperidol binding, we studied the condition in which neither ascorbate nor guanyl nucleotides were included, the condition in which only one of the agents was included, and the condition in which both agents were included. The first series of studies performed was rate kinetic studies. In the absence of either ascorbate and/or guanyl nucleotides, [^{3}H]spiroperidol rapidly associated to its binding sites in human

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TABLE 1

The two components of [^{3}H]spiroperidol binding in human caudate and putamen exhibit dopaminergic selectivity

Dose response studies were performed by placing increasing concentrations (at least 30) of the ligands shown, 0.2 nM [^{3}H]spiroperidol, 34.4 mM Na/Hepes (pH 7.4 at 37°), 3.4 mM $MgSO_4$, and 0.2 ± 0.05 mg of protein from human caudate particulate membrane fragment preparations in test tubes and incubating the reaction for 60 min at 37°. At least two studies for each drug were performed and co-analyzed by LIGAND (13) as stated under Materials and Methods. The kinetic parameters for [^{3}H]spiroperidol binding were derived from saturation analyses under the same conditions (as detailed below). In all cases a two-site fit with independent K_i was the preferred fit. Although not shown, the results in putamen were similar. K_H and K_L refer to the K_A of the competitor for the high and low affinity [^{3}H]spiroperidol-binding components, respectively. The K_i values are given in parentheses below each ligand. "With Ascorbate" refers to the same binding assay media with the addition of 0.1 mM ascorbate and an incubation of 30 min at 37°. The kinetic parameters for [^{3}H]spiroperidol binding used in these analyses were derived from saturation analyses performed under the same conditions (see below).

Drug	K_H	K_L
	M^{-1}	
Without ascorbate		
Domperidone	$5.0 \pm 2.3 \times 10^9$ (2.0×10^{-10} M)	$1.8 \pm 2.8 \times 10^7$ (5.4×10^{-8} M)
(±)-Sulpiride	$2.6 \pm 0.5 \times 10^7$ (3.8×10^{-8} M)	$2.2 \pm 6.1 \times 10^4$ (4.5×10^{-5} M)
Ketanserin	$3.8 \pm 1.5 \times 10^6$ (2.6×10^{-7} M)	$3.1 \pm 3.9 \times 10^6$ (3.3×10^{-7} M)
Dopamine	$2.4 \pm 1.8 \times 10^9$ (4.2×10^{-10} M)	$1.5 \pm 0.5 \times 10^6$ (6.7×10^{-7} M)
Serotonin	$6.0 \pm 0.9 \times 10^4$ (1.7×10^{-5} M)	$2.2 \pm 1.2 \times 10^5$ (4.5×10^{-6} M)
With ascorbate		
Dopamine	$4.4 \pm 4.8 \times 10^9$ (2.3×10^{-10} M)	$6.9 \pm 2.5 \times 10^5$ (1.4×10^{-6} M)
Serotonin	$8.2 \pm 5.3 \times 10^3$ (1.2×10^{-4} M)	$3.1 \pm 1.1 \times 10^5$ (3.2×10^{-6} M)

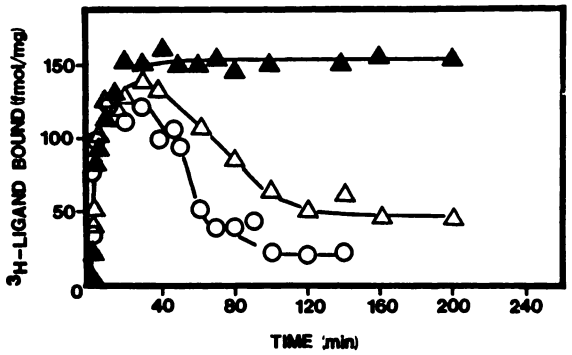

Fig. 1. The effect of ascorbate and GTP on the association of [^{3}H] spiroperidol to its binding sites in human caudate and putamen. Rate association studies were performed by placing 0.2 nM [^{3}H]spiroperidol, 0.2 ± 0.05 mg of protein from prepared particulate membrane fragments of human caudate or putamen, and 34.4 mM Na/Hepes (pH 7.4 at 37°) in test tubes and incubating the reaction at 37° for the times shown. Specific binding (shown) was determined as the difference between [^{3}H] spiroperidol binding in the absence and presence of 10 μM haloperidol at the time points shown. Nonspecific binding in each condition was essentially identical, and unchanged from 2 to 200 min (not shown). Three conditions were studied: ▲, no further additions were made; △, 0.1 mM ascorbate (final concentration) was present; and ○, 0.1 mM ascorbate and 1 mM GTP were present. A fourth condition in which GTP was present but ascorbate was not resulted in data superimposable on those shown for the no addition condition. Each study is an individual study on a single postmortem specimen that has been replicated at least three times with essentially similar results.

caudate and putamen, achieving an apparent steady state by at least 20 min which persisted unchanged for at least 200 min at 37° (Fig. 1). The addition of 0.1 mM ascorbate altered the apparent steady state. In the presence of ascorbate, the initial steady state persisted only until 50–60 min. At this time an apparent loss of specific [^{3}H]spiroperidol binding began to occur and lasted until a final steady state was reached by 120 min (Fig. 1). An appreciable amount of specific binding (>40% in all studies, $N = 18$) remained at this final steady state. The addition of 1 mM GTP without ascorbate did not change the association of [^{3}H]spiroperidol to its binding sites (not shown, but superimposable on the control study in Fig. 1). However, in the presence of 0.1 mM ascorbate *and* 1 mM GTP, the initial steady state was quite abbreviated, the apparent loss of binding was more rapid, and by 120 min incubation, there was >85% loss of specific [^{3}H]spiroperidol binding (Fig. 1). Therefore, both the rate and the magnitude of ascorbate-induced losses of binding were enhanced by the addition of GTP.

The second series of studies performed was saturation studies. The primary purpose of these studies was to determine whether a two-site fit was preferred under control conditions, in the presence of ascorbate, or in the presence of ascorbate plus GTP at the steady states observed in the above studies. In the absence of ascorbate and GTP, saturation isotherms of [^{3}H] spiroperidol binding in human caudate or putamen were best fit by a two-site model (Fig. 2, *left*, and Table 2) whether performed at 30 min, 60 min, or 120 min incubation (only the

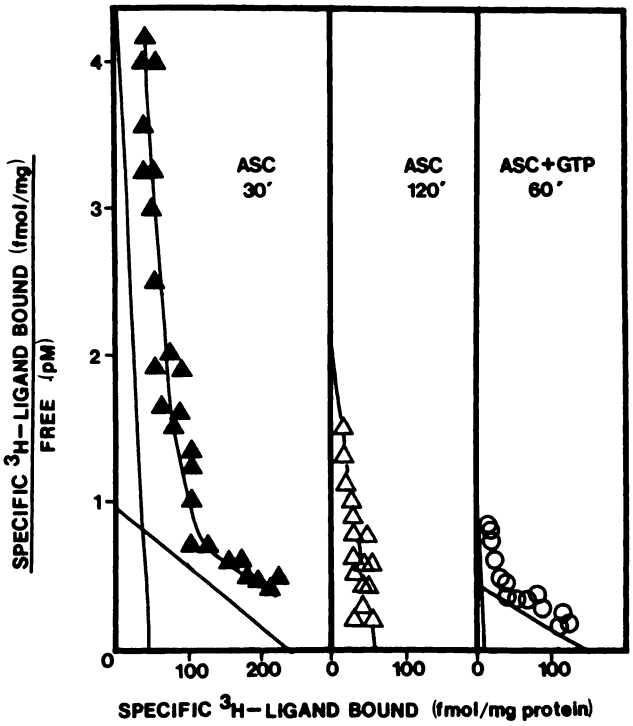

Fig. 2. The effects of ascorbate (*ASC*) and ascorbate plus GTP on saturation isotherms of [^{3}H]spiroperidol binding in human caudate and putamen. Saturation studies were performed by placing increasing concentrations of [^{3}H]spiroperidol (0.01–5 nM) in test tubes with Na/Hepes and protein as in the text. *Left*. No further additions were made and an incubation time of 30 min at 37° was used. The *lines* shown correspond to the kinetic parameters derived from the computer-assisted analysis of this and its replicate studies as given in Table 2. *Middle*. Ascorbate was added for a final concentration of 0.1 mM, and an incubation time of 120 min at 37° was used. These studies also contained 3.4 mM $MgSO_4$ which itself has no appreciable effect on [^{3}H]spiroperidol binding (not shown). *Right*. Both 1 mM GTP and 0.1 mM ascorbate were added, and the incubation was for 100 min at 37°. Nonspecific binding was determined as described in the text. An individual experiment is shown for each condition and the results of the computer-assisted analyses of these and the replicate data are shown in Table 2.

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TABLE 2

The kinetic parameters of [^{3}H]spiroperidol binding in the absence of ascorbate or GTP and in the presence of ascorbate, GTP, or ascorbate plus GTP.

Saturation studies were performed as stated in the legend to Fig. 2, in the presence of each addition shown. The studies performed in the presence of ascorbate at 30 min and 120 min also contained 3.4 mM $MgSO_4$. The parameters shown were derived from the preferred fit determined by LIGAND (15) as stated under Materials and Methods. A minimum of two experiments with 22 points each were analyzed for each condition.

	K_{Ahigh}	K_{ALow}	$B_{maxHigh}$	B_{maxLow}
	$\times 10^{10}$ M^{-1}	$\times 10^{9}$ M^{-1}	fmol/mg	
No additions				
Caudate	9.4 ± 4.3 (1.1×10^{-11} M)	3.6 ± 4.9 (2.8×10^{-10} M)	80 ± 25	105 ± 55
Ascorbate (30 min)				
Caudate	13 ± 20 (7.6×10^{-12} M)	4.0 ± 2.9 (2.5×10^{-10} M)	47 ± 36	250 ± 41
Putamen	6.0 ± 8.8 (1.6×10^{-11} M)	3.0 ± 3.9 (3.3×10^{-10} M)	50 ± 69	340 ± 120
Ascorbate (120 min)				
Caudate	5.0 ± 2.5 (2.0×10^{-11} M)		25 ± 8.1	
Putamen	3.6 ± 1.5 (2.8×10^{-11} M)		54 ± 14	
Ascorbate plus GTP (60 min)				
Caudate	7.3 ± 3.4 (1.4×10^{-11} M)	1.8 ± 0.4 (5.0×10^{-10} M)	16 ± 4.5	270 ± 46
GTP (100 min)				
Caudate	5.8 ± 5.5 (1.7×10^{-11} M)	3.8 ± 1.8 (2.6×10^{-10} M)	55 ± 38	230 ± 31

60-min incubation is shown). The addition of 0.1 mM ascorbate for 30 min incubation (initial steady state, Fig. 1) did not change the fact that a two-site fit was preferred (Table 2). However, if the saturation studies were performed at the final steady state in the presence of ascorbate (120 min incubation), the results were best fit by a one-site model. The presence of ascorbate for 120 min caused an apparent total loss of the *lower* affinity component of [^{3}H]spiroperidol binding (Fig. 2, Table 2). Saturation studies performed in the presence of GTP without ascorbate were best fit by a two-site model (Table 2), even when incubation times as long as 100 min were used. In an attempt to study a steady state condition for GTP plus ascorbate, an incubation time of 60 min was selected to avoid the times of most rapid change for either ascorbate alone or ascorbate plus GTP, as shown above. Under these conditions, a two-site model was preferred, but as shown in Fig. 2 and Table 2, there was an apparent reduction in the density of the highest affinity state for [^{3}H]spiroperidol, as well. It was clear from the rate kinetic studies that, in the presence of ascorbate plus GTP, both binding components were essentially lost at the final steady state, in that <15% specific [^{3}H]spiroperidol binding could be detected at 120 min incubation. With such low levels of detectable specific binding, saturation studies could not be performed under these conditions.

The effect of ascorbate and GTP on lipid peroxidation in human caudate and putamen. It had been reported previously that ascorbate induced lipid peroxidation in lower mammalian striatum and that this event was associated with a loss of specific [^{3}H]spiroperidol binding (8). We therefore determined whether ascorbate did induce lipid peroxidation in human caudate and putamen by measuring the production of TBARs under our standard assay conditions. Significant TBAR production was noted in the assay after 120 min incubation with ascorbate (Table 3). The amount of TBAR production was enhanced by the addition of iron and reduced by the

TABLE 3

The ability of ascorbate to induce lipid peroxidation in human caudate and putamen

Test tubes containing 34.4 mM Na/Hepes (pH 7.4 at the temperature of incubation), 0.1 mM ascorbate, additions as shown, and 0.2 ± 0.05 mg of protein/ml of assay (prepared particulate membrane fragments of human caudate or putamen) were incubated for 120 min at 37°, before TCA precipitation and addition of standard ingredients for the TBAR assay, as stated in the text. TBARs were determined in the presence or absence of ascorbate with the additions shown. The results shown are the mean ± standard deviation of the *N* replicates.

Ascorbate (0.1 mM)	Other additions	TBAR	*N*
		nmol MDA/mg protein	
−	None	0.0	4
−	3 μM $FeSO_4$	1.1 ± 0.5	4
−	10 μM EDTA	0.0	4
+	None	13 ± 4.1	9
+	3 μM $FeSO_4$	29 ± 13	8
+	10 μM EDTA	1.0 ± 0.6	5

presence of EDTA (Table 3). The effect of ascorbate on lipid peroxidation was nearly 30-fold greater than the effect of iron alone (Table 3). No TBARs were formed in the presence of assay ingredients without the addition of ascorbate or iron. The effect of ascorbate on TBAR production was somewhat idiosyncratic. The TBARs produced by incubation with ascorbate for 120 min at 37° in putamen from 13 postmortem specimens had a mean value of 12.1 ± 6.4 nmol of MDA/mg of protein, but the range was from 2.1 to 22.6 nmol of MDA/mg of protein. There was no correlation between TBARs produced and age ($r = -0.26$), postmortem interval ($r = -0.20$), or months for which the tissue had been stored <1 year ($r = 0.26$). However, there was a correlation between TBARs produced and months the tissue had been stored from 1 to 52 months ($r = -0.872$ and $p = 0.01$). Therefore, only tissue samples which had been stored <12 months were used for the studies reported here.

Ascorbate-induced lipid peroxidation occurred in a time-dependent fashion (Fig. 3) that was not unlike the observed

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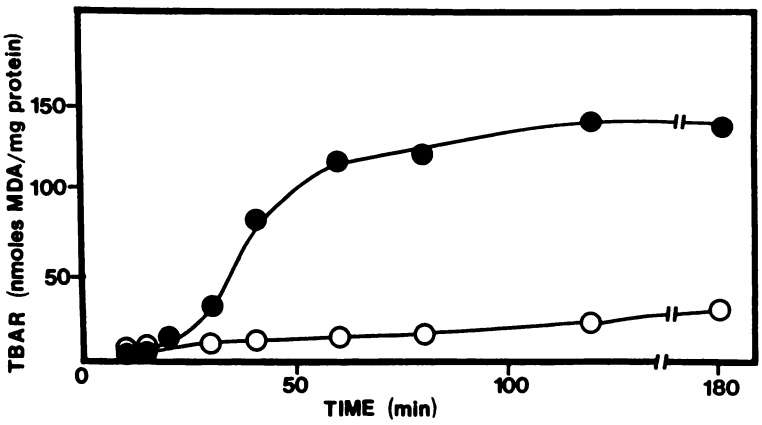

Fig. 3. GTP enhances ascorbate-induced lipid peroxidation. Test tubes, containing 0.1 mM ascorbate, 34.4 mM Na/Hepes (pH 7.4 at 37°), and 0.2 mg of protein/ml assay (human putamen particulate membrane fragments) with (●) or without (○) 1.0 mM GTP, were incubated for the times shown prior to TCA precipitation and the determination of TBARs as stated in the text. Both studies were done in tandem on the same postmortem specimen. The results are similar to three replicates on other postmortem specimens.

time course for the loss of specific [^{3}H]spiroperidol binding. When GTP was added with the ascorbate, lipid peroxidation occurred to a greater extent, and at an apparently more rapid rate (Fig. 3). This could correspond to the observation that both the rate and the magnitude of the ascorbate-induced loss of [^{3}H]spiroperidol binding were enhanced with the addition of GTP (Fig. 1). On the average, at a single time point (120 min at 37°), GTP plus ascorbate produced 3 times the amount of TBARs produced by ascorbate alone (Table 4). ITP was as effective as GTP in enhancing ascorbate-induced TBARs. Both IMP and GMP were substantially less able to enhance the ascorbate-induced effect, and ATP did not enhance the effect at all (Table 4). cAMP totally prevented the action of ascorbate in inducing TBAR production (Table 4). NaF and forskolin neither enhanced nor blocked ascorbate-induced TBAR production (Table 4). None of the nucleotides or NaF or forskolin produced TBARs in the absence of ascorbate (not shown).

The iron chelator desferrioxamine (10 μM) and the antioxidant DTT at 1 mM prevented TBAR production in the presence of either ascorbate or ascorbate plus GTP (Table 5). Dose response studies of the desferrioxamine inhibition of ascorbate-induced TBAR production resulted in an IC_{50} of $4.1 \pm 1.1 \times 10^{-7}$ M ($N = 5$). The same studies of GTP plus ascorbate-induced TBAR production resulted in an IC_{50} of $1.3 \pm 1.2 \times 10^{-7}$ M. Dopamine, at 100 μM, reversed the effects of both ascorbate-induced lipid peroxidation and GTP enhancement of ascorbate-induced lipid peroxidation (Table 5).

We next examined the association between the loss of binding and ascorbate-induced TBAR formation. We first examined whether ascorbate-induced TBARs were correlated with the ascorbate-induced loss of binding, when the loss of binding was determined as the difference in binding in the presence and absence of ascorbate at 0.2 nM [^{3}H]spiroperidol and 120 min incubation at 37°. The loss of binding did correlate with TBAR production (as determined by least squares linear regression) at $r = 0.62$ ($N = 10$), which was significant (as determined by Student's t test) at $p = 0.05$. Second, we examined the effects of desferrioxamine and the antioxidant Trolox (a water-soluble antioxidant of equivalent efficacy to α-tocopherol). As shown in Table 6, just as desferrioxamine and antioxidants prevented TBAR formation in the presence of ascorbate, they also prevented the loss of binding induced by ascorbate.

A relatively recent report demonstrated that aldehydes, oxidized in the presence of TBA, produced an unstable yellow pigment with maximal absorbance at 450 nm (16). Esterbauer (17) had shown that oxidation of aldehydes and other indications of lipid peroxidation could be observed in the absence of

TABLE 4

The effects of nucleotides, NaF, and forskolin on ascorbate-induced lipid peroxidation as measured by TBAR production in human caudate and putamen

TBARs were determined as stated in the text. The amount of TBARs (nmol of MDA produced) in the presence of 0.1 mM ascorbate was considered 100% TBARs. Shown are the mean ± standard deviation of the N replicates.

Addition	Concentration	Percentage of ascorbate-induced TBARs	N
	mM		
GTP	1	327.3 ± 95.3	11
ITP	1	360.4 ± 70.6	3
GMP	1	115.6 ± 12.2	3
IMP	1	133.0 ± 2.4	3
ATP	1	103.9 ± 43.0	3
cAMP	1	2.0 ± 3.4	3
NaF	10	104.4 ± 18.1	3
Forskolin	0.01	99.6 ± 18.5	3

TABLE 5

The effects of antioxidants and other agents on ascorbate- and GTP plus ascorbate-induced TBAR production in human caudate and putamen

The TBAR assay was performed as stated in the text, in the presence of 0.1 mM ascorbate, or 0.1 mM ascorbate plus 1.0 mM GTP with additions as shown. Shown are the mean ± standard deviation of the number of replicates shown in parentheses.

Addition	Percentage of TBARs produced	
	(+) Ascorbate	(+) Ascorbate plus GTP
None	100	100
Desferrioxamine (10 μM)	0.0 ± 0.0 (5)	3.6 ± 1.6 (5)
DTT (1 mM)	7.8 ± 1.1 (3)	5.0 ± 1.8 (3)
Dopamine	0.0 ± 0.0 (4)	1.3 ± 0.5 (3)

TABLE 6

The effects of iron chelators and antioxidants on ascorbate-induced losses of binding

Specific [^{3}H]spiroperidol binding was determined at 0.2 nM [^{3}H]spiroperidol in the presence of 0.1 mM ascorbate. Specific binding at 30 min was considered maximal binding. Binding at 120 min/binding at 30 min is shown. The dose of Trolox and desferrioxamine used had no effect on binding at 30 min (not shown). Shown are the mean ± standard deviation of the number of replicates shown in parentheses. Differences between means and control values (ascorbate alone) are all statistically significant at $p = 0.01$.

Addition	Specific binding at 120 min/30 min
	%
Ascorbate (0.1 mM)	68.5 ± 5.2 (7)
Ascorbate + 10 μM desferioxamine	95.0 ± 11 (4)
Ascorbate + 10 μM Trolox	97.5 ± 9.0 (4)

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the production of TBARs. We therefore measured absorbance at 450 nm under several conditions, some of which were similar to binding assay conditions used by other investigators. Absorbance at 450 nm did not vary with the amount of TBA added in the absence of tissue, and in the presence of tissue a stable background absorbance could be measured (not shown). As shown in Table 7, incubation of tissue with 0.1 mM ascorbate and 1 mM ITP at 37° for 120 min produced a detectable increase in absorbance at 450 nm over background (tissue alone). It should be noted that neither ascorbate nor ITP increased absorbance at 450 nm in the absence of tissue. At 120 min incubation at 37°, a substantial amount of absorbance at 532 nm was also present, representing TBARs (Table 4). Incubation of tissue for 15 min at 37° with 5.7 mM (0.1%) ascorbate, 1 mM ITP, and 1 mM EDTA produced no measurable absorbance at 532 nm (hence no TBARs) but produced a detectable increase in absorbance at 450 nm (Table 7). Incubation of tissue with the same ingredients for 60 min at 25° also resulted in an increased absorbance at 450 nm.

The effect of guanyl nucleotides on agonist binding in the absence of ascorbate. Dose response studies performed in the presence of 1.0 mM GTP without ascorbate were best fit by a two-site model with independent K_i (Table 8). As compared to the studies in the absence of GTP, where the K_i values of dopamine were 0.4 and 660 nM, the K_i values in the presence of GTP were increased to 24 and 300,000 nM, respectively (Table 8). The percentage of high and low affinity states for dopamine in the presence of GTP (Table 8) remained within the range of those predicted from saturation studies in the absence of GTP. The percentages of high and low affinity [^{3}H] spiroperidol binding components at the concentration of [^{3}H] spiroperidol used for the dose response studies were determined from the saturation studies on all postmortem specimens in the absence of ascorbate and were 10–40% for the highest affinity state and 60–90% for the lowest affinity state.

TABLE 7

Evidence of lipid peroxidation occurs under some assay conditions in the absence of TBAR formation

The TBAR assay was performed as stated under Materials and Methods and absorbance was determined at both 450 nm and 532 nm. "Addition" refers to the presence of any additions beyond buffer and tissue. The additions included ascorbate (ASC), ITP (at 1.0 mM), and/or EDTA (at 1.0 mM). T° refers to the temperature of the assay and "Time" to the incubation time. The presence of absorbance at 450 nm was determined as the percentage increase above background (BKG). The presence of absorbance at 532 nm, representing TBARs, is shown as present (+) or absent (–). The results shown are from three experiments in each condition.

Addition	T°	Time	% Increase over BKG at 450 nm	Absorbance at 532 nm
	°C	min		
0.1 mM ASC	37	120	14.6 ± 1.5	+
5.7 mM ASC/ITP/EDTA	37	15	23.9 ± 12.7	–
5.7 mM ASC/ITP/EDTA	25	60	27.7 ± 5.1	–

TABLE 8

The effect of GTP in the absence of ascorbate on dopamine inhibition of [^{3}H]spiroperidol binding

Dose response studies were performed at 0.2 nM [^{3}H]spiroperidol in the absence of added ascorbate. Dopamine was added in increasing concentrations from 1 pM to 1 mM (at least 35 concentrations were studied in each experiment). Shown is the best fit of the data obtained by computer-assisted analysis of two dose response studies in the absence of GTP and in the presence of 1.0 mM GTP. Again, four postmortem specimens were used for the study. The K_i values are given in parentheses.

	K_{Ahigh} (M^{-1})	K_{ALow} (M^{-1})	% R High	% R Low
No GTP	$2.4 \pm 1.8 \times 10^9$ (4.2×10^{-10} M)	$1.5 \pm 0.5 \times 10^6$ (6.7×10^{-7} M)	18	82
+ GTP	$4.2 \pm 2.7 \times 10^7$ (2.4×10^{-8} M)	$3.3 \pm 1.3 \times 10^3$ (3.0×10^{-4} M)	10	90

Discussion

Ascorbate induces a loss of specific [^{3}H]spiroperidol binding in human caudate and putamen leaving no detectable binding at the lowest affinity component of [^{3}H]spiroperidol binding by 120 min, at which time appreciable amounts of binding at the highest affinity component of [^{3}H]spiroperidol binding remain. The addition of GTP to ascorbate-containing media increases both the rate and the magnitude of the loss of binding such that a nearly total loss of binding, affecting both the high and low affinity components, occurs by 120 min incubation. At incubation times less than 120 min, intermediate conditions, with appreciable losses of the highest affinity component of [^{3}H]spiroperidol binding, are observed. Ascorbate increases the concentration of the products of lipid peroxidation in these tissues, and the rate and magnitude of product formation are increased by the addition of GTP to ascorbate-containing media. The lipid peroxidation and the loss of binding induced by ascorbate alone are associated in time and are both blocked by the presence of antioxidants and iron chelators. These findings could explain the discrepancies reported in the literature concerning the effect of GTP on agonist binding. Some investigators have reported that GTP promotes an interconversion of high affinity states for agonist to low affinity states for agonist. These investigators added ascorbate to the assay, and/or used tissue that had not been extensively washed in preparation (1–5). Ascorbate concentrations approach mM in the brain; therefore, the less extensively washed tissue may have added appreciable amounts of ascorbate or endogenous iron to the assay (18). In contrast, in another report, GTP was found to reduce agonist affinity at both [^{3}H]spiroperidol affinity states without any evidence for interconversion of agonist affinity states (6). These investigators omitted ascorbate and added DTT to the assay. The data presented here demonstrate that GTP, in the presence of ascorbate, causes an accelerated loss of [^{3}H]spiroperidol binding compared to ascorbate alone. GTP itself, in the absence of ascorbate, causes a dramatic reduction in the affinity of agonists for both [^{3}H]spiroperidol binding sites in human caudate and putamen (Table 8) without affecting radiolabeled antagonist binding. There is no apparent interconversion of agonist affinity states in the absence of ascorbate. Perhaps, then, in the presence of GTP and ascorbate an accelerated loss of the highest affinity component of radiolabeled antagonist binding has occurred, which, studied from the perspective of agonist competition, would appear to represent "interconversion" of a high affinity state for agonist into the remaining lower affinity state for both agonist and antagonist. In contrast, in the absence of ascorbate, no appreciable loss of high affinity binding occurs and, therefore, no "interconversion" of affinity states is apparent. This concept is supported by the observation that both lipid peroxidation and "interconversion" of affinity states for dopamine are temperature dependent, with neither occurring at 4° (5, 8).

There are two high affinity [^{3}H]spiroperidol-binding sites present in human caudate and putamen, as previously reported (14, 15) and presented here. Both of these affinity states exhibit a higher affinity for dopamine than serotonin. The serotonin antagonist, ketanserin, has a K_i at each of these affinity states

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consistent with its K_i at dopamine, rather than serotonergic binding sites (19). The majority of the selectivity studies are consistent with the hypothesis that both of these [^{3}H]spiroperidol affinity states are dopaminergic, even though the results with domperidone and (±)-sulpiride suggest K_i values consistent with binding at a dopaminergic and a serotonergic site (19, 20). Attempts to perform saturation analyses in the presence of 10 μM (±)-sulpiride resulted in <20% specific binding at all concentrations of radioligand studied, suggesting that a very small proportion, if any, of the human basal ganglia [^{3}H] spiroperidol-binding sites are serotonergic. If both affinity states are not optimally studied, intermediary situations, such as a single set of sites with an intermediate K_D, or failure to recognize the dual components of antagonist binding, may occur.

Ascorbate induces a relatively slow loss of specific [^{3}H] spiroperidol binding in this tissue which renders the lowest affinity component of [^{3}H]spiroperidol binding undetectable by 120 min incubation at 37°. This loss is coincidental in time with ascorbate-induced lipid peroxidation. There is a positive correlation between the amount of binding lost and the amount of TBARs produced at 120 min incubation in the presence of ascorbate. The nucleotide profile (ITP = GTP > IMP = GMP >>> ATP) observed in the enhancement of ascorbate-induced lipid peroxidation is similar to the profile observed in the induction of a loss of [^{3}H]spiroperidol binding in rat striatum (7). Iron chelators and antioxidants block both the ascorbate-induced production of TBARs and the ascorbate-induced loss of binding. These factors suggest an association, although potentially spurious, between the events of the loss of binding and lipid peroxidation. The exact relationship and the possible mechanisms by which these events occur are under current investigation. The ascorbate-induced lipid peroxidation occurs at a faster rate in the presence of GTP, although GTP alone does not induce lipid peroxidation. In the presence of GTP and ascorbate, there is no appreciably detectable [^{3}H]spiroperidol binding at either high or low affinity components by 120 min incubation. Therefore, depending upon the time of incubation, the extent of washing in the initial tissue preparation, and the concentration of ascorbate added, more or less complete detection of both [^{3}H]spiroperidol affinity states could occur. Indeed, such discrepancies have been reported with regard to [^{3}H] spiroperidol-binding sites in pituitary or basal ganglia/striatum. Some investigators report two affinity states with K_D values ranging from 20 to 90 pM and from 0.1 to 4 nM for the highest and lowest affinity state, respectively (7, 15, 21–24). Other investigators report one affinity state with K_D values ranging from 100 to 800 pM (1–3, 5, 25–27). Those investigators observing two affinity states had several common features in their methodology: more than two washings of the tissue in the initial preparation with or without the addition of ascorbate in the assay; or less extensively washed tissue and no ascorbate in the assay; and/or increased volumes of assay at low radioligand concentrations. Those investigators observing only one affinity state had the following features in common: twice washed tissue with or without ascorbate in the assay; and/or an incubation period for the homogenized tissue at 37°. We have previously demonstrated that the number of washings is a critical factor in the detection of the highest affinity state in rat striatum (22). Since GTP concentrations approach mM in the brain, at least a 1000-fold dilution during the washing procedure would be required to reduce GTP to μM concentrations or less. Therefore, in less extensively washed tissue, in the presence of ascorbate (either added or present in the tissue), the endogenous GTP could reduce the density of the highest affinity component of [^{3}H]spiroperidol binding. At the various temperatures and incubation times used by investigators, this effect of GTP could be more or less complete. Additionally, since all these studies were performed with ^{3}H-ligands, unless the assay and assay constituent volumes were increased at low radioligand concentrations (<0.1 nM), conditions for the detection of the highest affinity state might not be optimal. Therefore, if the density of that affinity state were reduced by the presence of endogenous GTP, ascorbate, and iron, that affinity state might not be detected at all in saturation studies with radiolabeled antagonist.

Whether 5.7 mM or 0.1 mM ascorbate, lowered temperatures, or shorter incubation times at 37° are used, and whether or not EDTA is present, some absorbance at 450 nm is produced in the standard TBAR assay, even if absorbance at 532 nM (TBAR) is not appreciably increased. The increased absorbance at 450 nm is most likely indicative of the production of an unstable yellow pigment related to the oxidation of aldehydes. The oxidation of aldehydes can be an indicator of lipid peroxidation even in the absence of a production of TBARs, as Esterbauer (17) has demonstrated. Therefore, lipid peroxidation is occurring in the presence of guanyl or inosine nucleotides in all of the commonly used assay conditions. The mechanism by which the lipid peroxidation is occurring and whether the mechanism is different in the presence of ascorbate or ascorbate plus nucleotides, is beyond the scope of this study but is under current investigation. Nonetheless, the associations shown above demonstrate that it is probable that a loss of [^{3}H] spiroperidol-binding sites will occur in the presence of lipid peroxidation. Therefore, assay conditions in which any products of lipid peroxidation can be detected (those at 450 nm or 532 nm) may be compromising the population of [^{3}H]spiroperidol-binding sites.

Finally, our studies were performed in broken cell preparations, and whole cells may behave differently. However, Martres *et al.* (28) have presented data indicating that ^{3}H-antagonist binding in striatal slices is similar to ^{3}H-antagonist binding in membranes. Furthermore, these authors noted that GTP reduced high affinity ^{3}H-antagonist binding in the presence of ascorbate in striatal slices as it did in our particulate membrane preparation (28). Ascorbate concentrations approach mM in the brain (18), and ascorbate may be released upon neuronal depolarization (29). GTP concentrations also approach mM. Therefore, it may be likely that a loss of high affinity [^{3}H]spiroperidol-binding sites is occurring and is thereby reducing the affinity of dopamine from its affinity at high and low affinity states to its affinity at low affinity states only. However, dopamine inhibits ascorbate-induced lipid peroxidation, as shown in Table 5 and as previously noted by Kohn and Liversedge (11). Dopamine concentrations can approach mM in the synapse. Therefore, it may also be likely that the physiological mechanism is a reduction of the affinity of dopamine at intact [^{3}H]spiroperidol-binding sites. Although knowing which mechanism is operative *in vivo* is important for the correct modeling of the [^{3}H]spiroperidol-labeled dopamine receptor, it appears that GTP may reduce the effect of dopamine at [^{3}H]spiroperidol-binding sites by reducing its affinity.

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Since we are not sure which, if either or both, of these two [^{3}H] spiroperidol binding sites is coupled to a physiologic response such as the inhibition of adenylate cyclase (30), we cannot be certain of the ultimate physiologic importance of this reduction in affinity. However, at the least, the complex regulation of synaptic concentrations of GTP, ascorbate, and dopamine could serve as an elaborate modulating system for the control of dopaminergic activity.

We conclude that: 1) there are two affinity states for [^{3}H] spiroperidol in striatum and basal ganglia, each of which have dopaminergic selectivity; 2) GTP itself decreases the affinity of dopamine at both of these sites by at least 60 orders of magnitude; 3) in the presence of ascorbate, either added or endogenous, there is a slow loss of binding which is associated with lipid peroxidation; and 4) GTP added to ascorbate, in the absence of iron chelators or antioxidants, accelerates this loss of binding such that both [^{3}H]spiroperidol binding components are compromised in association with an enhancement of ascorbate-induced lipid peroxidation. Therefore, the analysis of the effects of nucleotides on [^{3}H]spiroperidol-labeled dopamine receptors can be complicated by the concurrent interactive effects of assay ingredients on ligand binding at those receptors.

Acknowledgments

We are grateful for the expert technical assistance of Mr. Mark Franko.

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