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Ascorbate Destruction of Opiate Stereospecific Binding in Guinea Pig Brain Homogenate

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SUMMARY

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Ascorbic acid was found to destroy opioid stereospecific binding to guinea pig brain homogenate irreversibly. This inactivation of stereospecific binding (SSB) displayed two components: a rapid phase that destroyed up to 50% of the binding within one minute, and a slower phase that proceeded by pseudo-first order kinetics. The ascorbate destruction of opioid binding was pH dependent, and was manifested primarily as a reduction in the number of binding sites, as demonstrated by Scatchard analysis. The slow phase of inactivation was subject to autoinhibition by high concentrations of ascorbate. Destruction of stereospecific binding by direct chemical reduction was ruled out, since several other reducing agents were found to be without effect. The stereoisomer of ascorbic acid, D-isoascorbic acid, produced destruction of SSB similar to that caused by ascorbate. Dehydroascorbic acid produced only slight loss of SSB, and protected against further destruction by ascorbic acid. All other analogues tested produced neither destruction of SSB nor protection against destruction of SSB by ascorbate. Destruction of stereospecific binding was prevented in homogenates incubated with ascorbate in the absence of oxygen. Reagents that inhibit ascorbate catalyzed lipid peroxide formation were shown to inhibit ascorbate destruction of SSB also, and dose-response curves for ascorbate destruction of SSB paralleled those for ascorbate-induced lipid peroxidation. Stereospecific binding could be partially but significantly protected by preincubation with phosphatidyl serine, but not with phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl inositol, cholesterol, cerebroside sulfates, sphingomyelin, arachidonic acid, or docosahexaenoic acid. It is suggested that a membrane lipid, sensitive to ascorbate induced peroxidation, plays a critical role in the structural integrity of the opioid stereospecfiic binding site.

INTRODUCTION

In 1971, Goldstein, Lowney, and Pal (1) emphasized the requirement of stereospecificity for opiate receptor binding, and demonstrated a small fraction of total opiate binding in mouse brain homogenates to be stereospecific. Three independent groups (2–4) soon developed assays in which the majority of the total opiate binding was shown to be stereospecific. Stereospecific binding of opiates has been shown to be destroyed by proteolytic enzymes (5, 6) and by treatment with phospholipase A_2 (5, 7, 8), suggesting a role for both protein and lipid in the binding function of the receptor.

Recently it was reported that ascorbic

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acid was successfully used as a treatment for heroin addiction (9). Since ascorbic acid is known to interact with many enzyme systems and biochemical pathways, this reagent was tested in the guinea pig brain homogenate opioid binding assay to determine what effects, if any, it had on opiate stereospecific binding. In this paper we report that ascorbate destroys opiate stereospecific binding through an oxygen dependent mechanism.

MATERIALS

L-Ascorbic acid used in this investigation was purchased from either Sigma Chemical Co. (St. Louis, Mo.) or J.T. Baker Chemical Co. (Phillipsburg, N.J). Stock solutions of either 1 M ascorbic acid or 1 M Tris-ascorbate were used interchangeably to prepare experimental solutions of ascorbate up to 1 mM in concentration. Tris-ascorbate solutions were prepared by neutralizing equimolar amounts of ascorbic acid with Trizma base obtained from Sigma, and were used in all experiments which required ascorbate concentrations greater than 1 mM. Stock solutions were freshly prepared every 5 to 7 days and kept refrigerated, with dilutions taken as needed.

[15,16(*N*)-^{3}H]etorphine at a specific activity of 32 Ci/mmole, and [tyrosyl-3,5-^{3}H]enkephalin (5-L-leucine), 41 Ci/mmole, were purchased from Amersham (Arlington Heights, Ill.). [^{3}H(G)]naloxone, 18.5 Ci/mmole, was obtained from New England Nuclear (Boston, Mass.). Levorphanol and dextrorphan were generous gifts from Hofmann La Roche (Nutley, N.J.).

D-Isoascorbic acid, L-ascorbic acid-2-sulfate (barium salt), glutathione (reduced form), acetylsalicylic acid, indomethacin, ouabain, L-α-phosphatidyl serine, L-α-phosphatidyl ethanolamine, DL-α-phosphatidyl choline, L-α-phosphatidyl inositol, cholesterol, sphingomyelin, arachidonic acid, and *N*-ethylmaleimide were obtained from Sigma Chemical Co. Sodium borohydride, potassium nitrite, calcium chloride, magnesium chloride, manganese (II) chloride, cobalt (II) chloride, 2-thiobarbituric acid, trichloroacetic acid, [ethylenebis (oxyethlenenitrilo)]tetraacetic acid (EGTA), and (ethylenedinitrilo)-tetraacetic acid (EDTA) were purchased from J. T. Baker Chemical Co.[2]

Dehydroascorbic acid was obtained from both ICN Pharmaceuticals (Plainview, N.Y.) and Pfaltz and Bauer, Inc. (Flushing, N.Y.); sulfatides (mixed cerebroside sulfates) and docosahexaenoic acid came from Applied Science Laboratories (State College, Pa.). Dithiothreitol, α-D-glucoheptonic acid-γ-lactone, and D-(+)-ribonic acid-γ-lactone were purchased from Aldrich Chemical Co. (Milwaukee, Wis.), and D-gulono-γ-lactone and L-galactono-1,4-lactone were obtained from Pfanstiehl Laboratories, Inc. (Waukegan, Ill.). Lanthanum (III) chloride was purchased from Matheson, Coleman, and Bell (Norwood, Ohio), chromium (III) chloride from Alfa Products (Danvers, Mass.), p-phenylene-diamine dihydrochloride (PPDA) from Accurate Chemical and Scientific Corp. (Hicksville, N.Y.), and 2-mercaptoethanol from Calbiochem (La Jolla, Ca.).

Carbon dioxide and carbon monoxide gases were obtained from Liquid Carbonic (Chicago, Ill.), and dry nitrogen from Matheson Gas Co. (Newark, Ca.). Male Simonsen-Hartley guinea pigs for the opiate radioreceptor binding assay were obtained from Simonsen Laboratories (Gilroy, Ca.).

METHODS

Guinea pig brain homogenate was prepared by the following procedure. A guinea pig was sacrificed by decapitation, and the brain quickly removed, dissected free of cerebellum, and washed in ice-cold 100 mM Tris-HCl, pH 7.4. The tissue was then homogenized in 10 volumes of Tris-HCl buffer using a teflon in glass mechanical homogenizer, and the homogenate diluted to 20 volumes with Tris-HCl buffer and centrifuged at 17,000 × *g* for 20 minutes. The pellet was resuspended in 20 volumes of ice-cold glass distilled water and allowed to stand on ice for 10 min to ensure lysis. After

[2] The abbreviations used are: SSB, stereospecific binding; EGTA, ethylenebis(oxyethlenenitrilo)-tetraacetic acid; EDTA, (ethylenedinitrilo)-tetraacetic acid; TLC, thin layer chromatography; NEM, *N*-ethylmaleimide; PPDA, paraphenylenediamine dihydrochloride; HPLC, high performance liquid chromatography.

centrifugation of the ice-water homogenate at 17,000 × *g* for 30 min, the pellet was resuspended in 100 mM Tris-HCl buffer to a final concentration of 2(w/v)%. Homogenate was either used immediately after preparation, or stored frozen for not more than one week.

The opiate radioligand binding assay was performed with 1% guinea pig brain homogenate in 100 mM Tris-HCl, pH 7.4, in a volume of 500 μl. Homogenate was subjected to various experimental manipulations, then incubated with radioligand in both the presence and absence of nonradioactive, competing ligand for 20 min at 22°. Following incubation, samples were placed on ice prior to filtration through Whatman GF-C glass fiber filters. [^{3}H]Etorphine was used at a final concentration of 2.5 nM, [^{3}H]leu-enkephalin and [^{3}H]naloxone at 8 nM, and competing ligands, levorphanol or levallorphan, at a final concentration of 1 μM, unless otherwise stated. The amount of radioligand displaced by the inactive stereoisomers, dextrorphan and dextrallorphan, was a small fraction of the total binding (less than 5%), and was disregarded. Stereospecific binding, therefore, was defined as the difference between radioligand binding in the absence and presence of competing, nonradioactive ligand.

Log-dose response curves were obtained by incubation of 1% homogenate with increasing concentrations of ascorbate for one hour at 22°, followed by direct addition of radioligand and assay for stereospecific binding. In all of the remaining experiments described, homogenates were washed free of ascorbate by two or more rounds of centrifugation for 10 min at 17,000 × *g* and resuspension in fresh, ice-cold Tris-HCl buffer prior to assay for stereospecific binding.

Details of homogenate treatments are given in the tables and figure legends where appropriate. For pH dependence studies, aliquots of homogenate were centrifuged at 17,000 × *g* for 10 min and resuspended in 100 mM Tris-HCl buffers, ranging from pH 7.0 to 8.2 with and without 1 mM ascorbate. All homogenates were incubated at 22° for one hour, washed, resuspended in pH 7.4 Tris-HCl, and assayed for SSB with [^{3}H]etorphine. Homogenates in buffers containing no ascorbate were used as controls for homogenates in buffers of matching pH which did contain ascorbate.

In studies of lipid protection against ascorbate destruction of opioid SSB, the various lipids tested were incorporated into homogenates using the procedure of Abood and Takeda (11). Appropriate amounts of lipids in chloroform-methanol solutions were evenly coated on the walls of 7 ml Dounce homogenizers by vortexing to dryness under a stream of nitrogen, followed by addition of 1 (w/v)% homogenate and vigorous homogenization. Lipids were demonstrated to be removed from the homogenizer walls by the absence of iodine staining in the homogenizers after the samples were removed. Aliquots of homogenates containing incorporated lipids were removed from the homogenizers and incubated for 15 min at 37° to allow temperature equilibration, followed by addition of ascorbate and further incubation for 15 min at 37°. After incubation, homogenates were washed free of ascorbate and unbound lipid and assayed for stereospecific binding. Lipid peroxide formation was determined by the procedure of Wilbur *et al.* (10). Lipid peroxide concentrations were calculated, employing a molar extinction coefficient of 1.56×10^5 determined from malonaldehyde standard curves.

Statistical analysis was performed using the Student's *t*-test for comparison of control and treated groups. Differences in variances for paired groups were checked for significance using the F statistic at $p = 0.05$.

RESULTS

Dose-response curves. Ascorbic acid was observed to inhibit opioid SSB, and dose-response curves were compiled for the amount of inhibition of [^{3}H]etorphine or [^{3}H]naloxone SSB produced in homogenates incubated with increasing concentrations of ascorbate for one hour at 22° both in the presence and absence of 100 mM NaCl (Fig. 1). The inhibition of [^{3}H]etorphine SSB was observed to be dose dependent over a concentration range from 1 μM to 1 mM ascorbate, with maximum inhibition (70–80% loss of control SSB) oc-

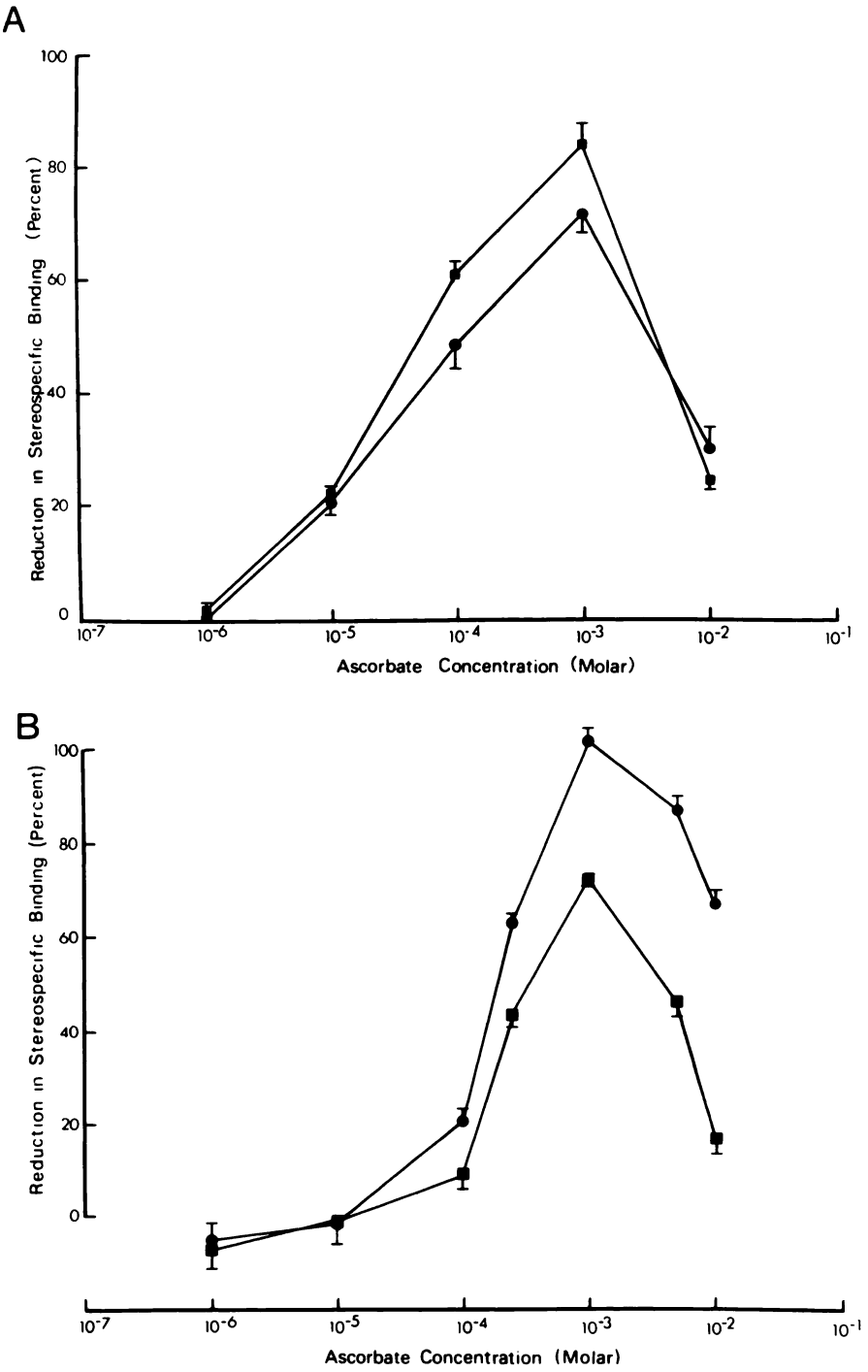

FIG. 1. *Dose-response curves for ascorbate inhibition of [^{3}H]etorphine and [^{3}H]naloxone stereospecific binding*

Panel A) [^{3}H]Etorphine:Homogenate was incubated for one hour at 22° with increasing concentrations of ascorbate in the presence or absence of 100 mM NaCl, followed by addition of 2.5 nM [^{3}H]etorphine and assay for SSB. Inhibition of [^{3}H]etorphine SSB expressed as percent reduction of control SSB is plotted against logarithm of molar ascorbate concentration in the absence (●) and presence (■) of NaCl. Each point represents the mean ± s.e.m. of four values. Panel B) [^{3}H]naloxone: Homogenate was incubated for one hour at 22° with increasing concentrations of ascorbate in the presence or absence of 100 mM NaCl, followed by addition of 8 nM ^{3}H-naloxone and assay for SSB. Inhibition of [^{3}H]naloxone SSB expressed as percent reduction of control SSB is plotted against logarithm of molar ascorbate concentration in the absence (●) and presence (■) of NaCl. Each point represents the mean ± s.e.m. of four values.

curing at 1 mM (Fig. 1a). Inhibition of [^{3}H]etorphine SSB in the presence of 100 mM NaCl was not significantly different from inhibition produced in the absence of NaCl. Dose-response curves in which [^{3}H]leu-enkephalin was used as radioligand were qualitatively almost identical to those for [^{3}H]etorphine (data not shown). Maximum inhibition of 88% of [^{3}H]leu-enkephalin SSB was achieved with 1 mM ascorbate, while 10 mM ascorbate inhibited only 30–40% of control [^{3}H]leu-enkephalin SSB. No chemical alteration of [^{3}H]etorphine or [^{3}H]leu-enkephalin could be detected by TLC when either ligand was incubated with ascorbate alone. Dose-response curves for ascorbate inhibition of [^{3}H]naloxone SSB were similar to those for [^{3}H]etorphine and [^{3}H]leucine-enkephalin, (Fig. 1b).

No recovery of SSB was observed in homogenates which had been washed up to four times by centrifugation, demonstrating that ascorbate produces an *irreversible* loss of SSB. In the remainder of the experiments presented here, homogenate preparations were washed free of ascorbate prior to assay for stereospecific binding.

Scatchard plots. Scatchard plots for [^{3}H]etorphine binding to homogenates previously treated with no ascorbate, 0.01 mM, 0.1 mM, 1 mM, and 20 mM ascorbate for 30 min at 22° to produce partial loss of SSB are shown in Fig. 2. In the control homogenate [^{3}H]etorphine exhibited a K_d of 0.24 nM, and the homogenate contained 14 fmoles stereospecific binding sites per mg of brain tissue. Incubation of homogenate with increasing ascorbate concentrations up to 1 mM produced a progressive decrease in the number of stereospecific binding sites. Treatment with 1 mM ascorbate, which gave maximum inactivation of stereospecific binding, reduced the number of stereospecific binding sites to 5 fmoles per mg tissue, with [^{3}H]etorphine K_d of 0.32 nM. Homogenate treated with 20 mM ascorbate contained 10 fmoles binding sites per mg tissue ([^{3}H]etorphine K_d = 0.24 nM).

Comparison of ascorbate and N-ethylmaleimide destruction of SSB. One percent guinea pig brain homogenate was treated with either 1 mM ascorbate or 1 mM NEM for 15 min at 37°. Both reagents produced about 87% destruction of control SSB (Ta-

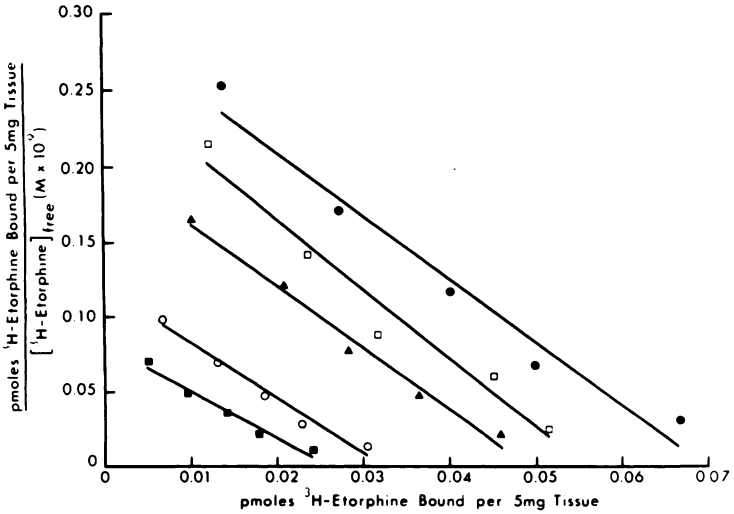

FIG. 2. *Scatchard plots for [^{3}H]etorphine stereospecific binding in guinea pig brain homogenate treated with increasing concentrations of ascorbate*

Homogenates were incubated for 30 min at 22° with no ascorbate (●), 0.01 mM ascorbate (□), 0.1 mM ascorbate (○), 1 mM ascorbate (■), and 20 mM ascorbate (▲), washed free of ascorbate by 2 rounds of centrifugation, and assayed for stereospecific binding with [^{3}H]etorphine at concentrations of 0.1 nM, 0.25 nM, 0.5 nM, 1 nM, and 2.5 nM. [^{3}H]Etorphine stereospecific binding in pmol/5 mg tissue divided by molar concentration of free [^{3}H]etorphine is plotted against [^{3}H]etorphine stereospecific binding (pmol/5 mg tissue) for each etorphine concentration employed.

ble 1). Pretreatment of homogenate with 1 μM levorphanol significantly decreased NEM destruction of SSB compared with dextrorphan pretreated homogenate (Student's t-test, $p < 0.001$). On the other hand, destruction of SSB by ascorbate in levorphanol pretreated homogenate was not significantly different from that in dextrorphan pretreated homogenate ($p > 0.1$).

Time course for ascorbate destruction of opioid stereospecific binding. When 1% guinea pig brain homogenate was incubated with various concentrations of ascorbate at 37°, two distinct phases of destruction of SSB were observed (Fig. 3). An initial, rapid phase of inactivation, complete in less than one minute (at which time the first samples were taken) was produced by all three concentrations of ascorbate employed. Following the initial rapid inactivation, a slower rate of destruction of SSB was observed with concentrations of 0.1 and 1 mM ascorbate, but not 20 mM ascorbate.

All three ascorbate concentrations destroyed approximately 50% of control [^{3}H]etorphine SSB by rapid inactivation (Table 2). The slow destruction of SSB produced by 0.1 and 1 mM ascorbate followed pseudo-first order kinetics, and rate constants and half-times for this destruction were obtained from the first order rate equation. The slow phase of [^{3}H]etorphine SSB destruction was practically eliminated by 20 mM ascorbate.

An Arrhenius plot for the slow inactivation of [^{3}H]etorphine SSB produced by 1 mM ascorbate at 0, 22, and 37° yielded a straight line with coefficient of determination (r^2) of 0.991 by linear regression analysis. The rate constant obtained for the reaction at 0° was 0.0014/min, with the other rate constants given in Table 2. From the Arrhenius plot, a value of 20 kcal/mol was obtained as the activation energy for the slow destruction of SSB by 1 mM ascorbate.

pH dependence and enzyme inhibitors. One millimolar ascorbate inactivation of etorphine SSB increased with increasing pH from 68% destruction of control SSB at pH 7.0 to a maximum of 85% at pH 7.8. The inactivation then decreased slightly and reached a plateau at 82% up to pH 8.2, the highest pH used. Several enzyme inhibitors, including sodium azide, potassium cyanide, ouabain, indomethacin, acetylsalicylic acid, and carbon monoxide were tested at concentrations up to 1 mM both for intrinsic effects on SSB and effects on ascorbate destruction of SSB. All inhibitors were found to be without effect either on SSB or on ascorbate induced destruction of SSB, except for indomethacin, which produced a small decrease in SSB by itself (data not shown).

TABLE 1

Comparison of ascorbate and N-ethylmaleimide produced destruction of [^{3}H]etorphine stereospecific binding to guinea pig brain homogenate, and protection against destruction by levorphanol and dextrorphan

One percent guinea pig brain homogenate was incubated with inactivating agent for 15 min at 37°. Pretreatment consisted of 15 min preincubation at 37° with protecting ligand. Following treatment, homogenates were washed free of ligands and reagents by 3 rounds of centrifugation and assayed for SSB with 2.5 nM [^{3}H]etorphine.

Values represent means and standard errors of three experiments expressed as percent of control stereospecific binding. Values for control SSB were 9.2, 10.4, and 14.3 fmol/mg tissue (wet weight) in the three experiments.

Inactivating agent	% Control SSB: no pretreatment	% Control SSB: 1 μM levorphanol pretreatment	% Control SSB: 1 μM dextrorphan pretreatment
Control	100 ± 14	120 ± 4	109 ± 10
1 mM *N*-ethylmaleimide	14 ± 2	88 ± 1	15 ± 2
1 mM ascorbate	12 ± 1	26 ± 4	17 ± 2

Reducing Agents. The effects of various reducing agents on opioid stereospecific binding in guinea pig brain homogenates are shown in Table 3. Ascorbic acid produced significant inactivation of control SSB both in the absence and presence of 100 mM NaCl ($p < 0.005$) and this loss of SSB was not recovered after washout of ascorbate. None of the other reducing agents tested had any effect on stereospecific binding.

Structure-activity-relationship studies.

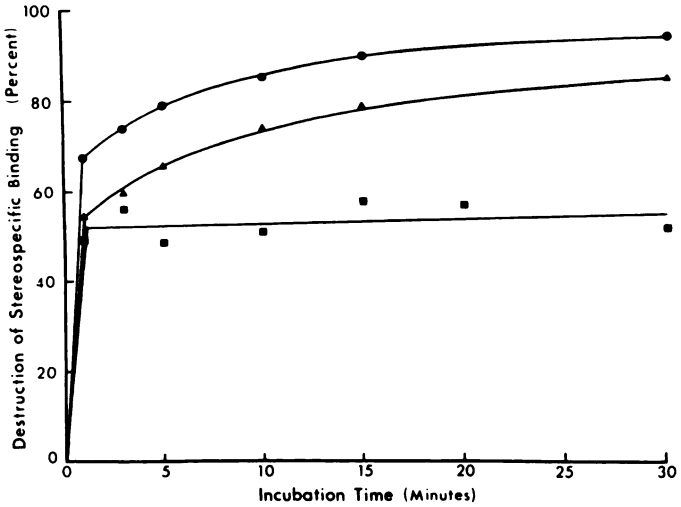

FIG. 3. *Time course for ascorbate destruction of [^{3}H]etorphine stereospecific binding in guinea pig brain homogenate at 37°*

Aliquots of control and ascorbate treated homogenates were removed at various time intervals, immediately diluted 1:4 in ice-cold Tris-HCl, washed free of ascorbate by 2 rounds of centrifugation and assayed for stereospecific binding with 2.5 nM [^{3}H]etorphine. Destruction of [^{3}H]etorphine stereospecific binding expressed as percent of control binding is plotted against incubation time in minutes with 0.1 mM ascorbate (▲), 1 mM ascorbate (●), and 20 mM ascorbate (■).

TABLE 2

Kinetic parameters for ascorbate inactivation of [^{3}H]etorphine stereospecific binding to 1% guinea pig brain homogenate at 22 and 37°

Each value represents the mean and standard error of three experiments.

Reaction conditions	Destruction of SSB (%) fast inactivation	k (min^{-1}) slow inactivation	$t_{1/2}$ (min) slow inactivation
0.1 mM Ascorbate 37°	53 ± 5	0.049 ± 0.004	14.3 ± 1.2
1.0 mM Ascorbate 37°	54 ± 7	0.115 ± 0.018	5.9 ± 1.5
20 mM Ascorbate 37°	44 ± 6	0.004 ± 0.002	260.8 ± 80.3
0.1 mM Ascorbate 22°	13 ± 2	0.012 ± 0.003	67.8 ± 17.0
1.0 mM Ascorbate 22°	27 ± 4	0.015 ± 0.003	53.0 ± 12.1
20 mM Ascorbate 22°	49 ± 4	0.0007 ± 0.0001	1071.0 ± 76.2

Several analogues of L-ascorbic acid, the structures of which are presented in Figure 4, were tested to determine both their intrinsic effects on SSB, and their effects on ascorbate destruction of SSB in guinea pig brain homogenate. The results are shown in Table 4. Destruction of control SSB by 1 mM D-isoascorbic acid (II) was highly significant ($p < 0.005$) and addition of 1 mM ascorbate neither increased nor decreased the destruction of SSB seen with D-isoascorbate. In five experiments, 1 mM dehydroascorbic acid reduced SSB significantly below control values ($p < 0.05$) and also gave significant protection against destruction of SSB by ascorbate ($p < 0.01$). All other analogues tested produced neither destruction of SSB nor protection against ascorbate induced destruction of SSB.

Anaerobic incubation. Very little difference was observed between SSB in control homogenates incubated under nitrogen and control homogenates incubated in contact with the atmosphere, as shown in Figure 5. However, there was a highly significant difference between residual SSB in homogenates treated with ascorbate under anaerobic conditions and under atmospheric con-

ditions ($p < 0.002$, $n = 3$). Ascorbate destruction of SSB was almost entirely prevented by removal of oxygen, and SSB in anaerobic control and ascorbate treated homogenates was not significantly different ($p > 0.2$).

Ascorbate induced lipid peroxidation. Homogenates were incubated with 1 mM ascorbate in the presence of various ions and reagents known to inhibit lipid peroxidation. In addition, several ions were tested that have no effect on lipid peroxidation. The results of these experiments are shown in Table 5. EDTA (100 μM), EGTA, (100 μM), PPDA (100 μM), Co^{+2} (10 μM), Cr^{+2} (100 μM), La^{+3} (100 μM) and Mn^{+2} (1 and 10 μM) all produced virtually complete inhibition of ascorbate induced lipid peroxide formation ($p < 0.005$). All of these reagents at the above concentrations also significantly reduced ascorbate destruction of SSB ($p < 0.001$). The effects of some metal ions, especially Cr^{+2} and La^{+3}, on both lipid peroxidation and SSB destruction were observed to be extremely concentration dependent. 100 μM Cr^{+2} produced highly significant reductions in both lipid peroxidation ($p < 0.005$) and SSB destruction ($p < 0.001$) from respective control values. 10 μM Cr^{+2} was much less effective in preventing ascorbate destruction of SSB and had no effect on lipid peroxide formation. The concentration dependence of La^{+3} ion was even more dramatic, with highly significant reduction of SSB destruction ($p < 0.001$) and lipid peroxide formation ($p < 0.005$) at 100 μM, but no significant decrease in either process at 10 μM. Neither 1 mM

TABLE 3

Effects of reducing agents on [^{3}H]etorphine stereospecific binding in guinea pig brain homogenate

One percent guinea pig brain homogenates were incubated with reducing agents (1 mM) in the presence and absence of 100 mM NaCl for one hour at 22°, then washed free of the agents by two rounds of centrifugation, resuspended to 1 (w/v)% in the appropriate buffer, and assayed for stereospecific binding with 2.5 mM [^{3}H]etorphine. Homogenates incubated with reducing agents in the presence of 100 mM NaCl were also assayed in the presence of 100 mM NaCl.

Each value represents the mean ± s.e.m. of three experiments.

Reducing agent	E_0' (volts, pH 7.0)	Percent inactivation of control stereospecific binding	
		No sodium	100 mM NaCl
Control homogenate	—	0 ± 8	0 ± 8
Potassium nitrite	0.42	−1 ± 4	−2 ± 4
Ascorbic acid	0.06	73 ± 4	75 ± 7
2-Mercaptoethanol	—	−15 ± 8	1 ± 7
Glutathione	−0.23	−6 ± 5	8 ± 4
Dithiothreitol	−0.33	11 ± 4	14 ± 4
Sodium borohydride	−1.24	−5 ± 7	3 ± 1

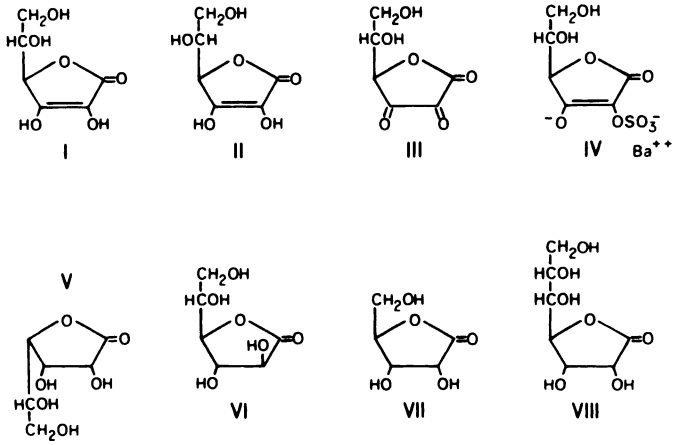

FIG. 4. *Structural analogues of* L-*ascorbic acid*

(I) L-ascorbic acid, (II) D-isoascorbic acid, (III) dehydroascorbic acid, (IV) L-ascorbic acid-2-sulfate (barium salt), (V) D-gulono-1,4-lactone, (VI) L-galactono-1,4-lactone, (VII) D-(+)-ribonic acid-γ-lactone, and (VIII) α-D-glucoheptonic acid-γ-lactone.

TABLE 4

Effects of structural analogues of ascorbic acid on [^{3}H]etorphine stereospecific binding and ascorbate induced destruction of stereospecific binding in guinea pig brain homogenate

One percent guinea pig brain homogenates were incubated with analogues (1 mM) in the absence (−Ascorbate) or presence (+Ascorbate) of 1 mM ascorbate, for one hour at 22°, then washed free of ascorbate and analogues by two rounds of centrifugation, resuspended to 1 (w/v)% in appropriate buffer, and assayed for [^{3}H]etorphine (2.5 nM) stereospecific binding.

Values represent means ± s.e.m. of (*n*) experiments.

Analogue	Percent inactivation of Control Stereospecific Binding	
	(−Ascorbate)	(+Ascorbate)
Control homogenate	0 ± 14 (9)	73 ± 3 (9)
D-Isoascorbic acid	77 ± 7 (3)	74 ± 6 (3)
Dehydroascorbic acid	25 ± 8 (5)	47 ± 9 (5)
L-Ascorbic acid-2-sulfate	4 ± 5 (3)	81 ± 2 (3)
D-Gulono-1,4-lactone	2 ± 4 (3)	77 ± 2 (3)
L-Galactono-1,4-lactone	4 ± 3 (3)	80 ± 8 (3)
D-(+)-Ribonic acid-γ-lactone	1 ± 1 (3)	71 ± 4 (3)
α-D-Glucoheptonic acid-γ-lactone	2 ± 3 (3)	76 ± 6 (3)

Ca^{+2} nor 1 mM Mg^{+2} had any effect on ascorbate induced lipid peroxidation or destruction of SSB. None of the reagents or ions, in the absence of ascorbate, had any effects on opioid SSB or lipid peroxide formation at the concentrations shown in Table 5. Analysis of a correlation plot of the data in Table 5 (SSB destruction vs lipid peroxide formation) yielded a correlation coefficient of 0.977.

Dose-response curves for ascorbate induced lipid peroxidation and ascorbate destruction of opioid stereospecific binding shown in Figure 6 were constructed from means ± s.e.m. of values from four experiments. These dose-response curves are almost superimposable over a concentration range from 10^{-5} to 10^{-3} M ascorbate. At 20 mM ascorbate, however, the two curves diverge. At this ascorbate concentration, lipid peroxidation is completely inhibited, while there is still a significant loss of stereospecific binding in ascorbate treated homogenate ($p < 0.05$). Ascorbic acid at 20 mM concentration had no effect on the thiobarbituric acid assay for lipid peroxide.

Lipid protection. Experiments were performed to determine if protection against ascorbate destruction of brain homogenate stereospecific binding could be provided by preincubation with biological lipids (Table 6). Incubation with each of the lipids, followed by washing of homogenate to remove free lipid, did not significantly alter stereospecific binding from control values, although there was a trend toward increased SSB with several of the lipids studied. Only phosphatidyl serine produced significant protection against destruction of SSB by 1 mM ascorbate.

Brain homogenates were incubated with

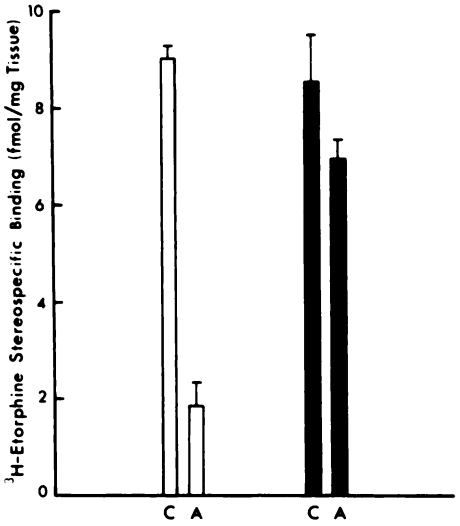

FIG. 5. *Effects of ascorbate on [^{3}H]etorphine stereospecific binding in guinea pig brain homogenate under anaerobic conditions*

Homogenates were degassed under vacuum, then half the homogenates were exposed to the atmosphere and the other half placed in a positive nitrogen atmosphere. Ascorbate at a final concentration of 1 mM was injected into each treatment flask through a rubber septum, and all homogenates incubated for one hour at 22°. At the end of the incubation aliquots from each homogenate were immediately diluted 1:4 in degassed, ice-cold Tris-HCl buffer, washed two times, and assayed for [^{3}H]etorphine stereospecific binding. Bar values presented are means ± s.e.m. of 3 experiments. Open bars represent homogenate kept in contact with the atmosphere, and solid bars represent degassed homogenate kept under a nitrogen atmosphere. C: control homogenate; A: ascorbate treated homogenate.

TABLE 5

Effects of chelating agents and metal ions on [^{3}H]-etorphine stereospecific binding and lipid peroxide formation in ascorbate treated homogenate

One percent guinea pig brain homogenates were incubated with ions or reagents in the presence of 1 mM ascorbate for one hour at 22°, then aliquots were removed and immediately assayed for either lipid peroxide formation, or assayed for stereospecific binding with 2.5 nM [^{3}H]etorphine.

Values represent means ± s.e.m. of (n) experiments.

Reagent	% of ascorbate induced destruction of stereospecific binding	% of ascorbate induced lipid peroxide formation
Control homogenate	100 ± 5 (11)	100 ± 14 (3)
10^{-4} M EDTA	−3 ± 5 (3)	−3 ± 2 (3)
10^{-4} M EGTA	−2 ± 5 (3)	0 ± 2 (3)
10^{-4} M PPDA	26 ± 4 (3)	14 ± 2 (3)
10^{-3} M Ca^{+2}	104 ± 7 (3)	98 ± 5 (3)
10^{-5} M Co^{+2}	2 ± 7 (4)	6 ± 2 (3)
10^{-6} M Co^{+2}	49 ± 6 (4)	44 ± 5 (3)
10^{-4} M Cr^{+2}	12 ± 7 (4)	8 ± 2 (3)
10^{-5} M Cr^{+2}	70 ± 8 (3)	88 ± 10 (3)
10^{-4} M La^{+3}	15 ± 13 (3)	−3 ± 2 (3)
10^{-5} M La^{+3}	103 ± 3 (3)	95 ± 9 (3)
10^{-3} M Mg^{+2}	88 ± 3 (3)	99 ± 4 (3)
10^{-5} M Mn^{+2}	−6 ± 6 (3)	−3 ± 6 (3)
10^{-6} M Mn^{+2}	21 ± 22 (3)	6 ± 6 (3)

1 mM and 20 mM ascorbate in the presence of 50 μg/ml phosphatidyl serine to determine if this lipid selectively protected against the slow or fast phase of ascorbate destruction of SSB (Fig. 7). Preincubation with phosphatidyl serine again gave significant protection against destruction by 1 mM ascorbate ($p < 0.05$), but offered no protection from destruction of SSB by 20 mM ascorbate.

Opiate bioassays. Ascorbic acid was tested in both guinea pig ileum-myenteric plexus (12) and mouse vas deferens (13) opiate bioassays and was found to be completely inert, displaying neither agonist nor antagonist properties. Sodium ascorbate had no effect on these preparations up to a concentration of 100 mM, which produced spasmogenic effects. However, when homogenates of guinea pig ileum were prepared for the opioid radioreceptor binding assay, opioid stereospecific binding in these homogenates was subject to complete inactivation by 1 mM ascorbate (unpublished data).

DISCUSSION

Log dose-response curves for ascorbate inhibition of opioid SSB were observed to be biphasic, with maximum inhibition of [^{3}H]etorphine, [^{3}H]leu-enkephalin, and [^{3}H]naloxone SSB at around 1 mM ascorbate, then decreasing up to 10 mM ascorbate. No differences were found for destruction of [^{3}H]etorphine and [^{3}H]leu-enkephalin SSB when homogenate was treated with ascorbate either in the absence or presence of 100 mM NaCl. Maximum inhibition of [^{3}H]naloxone was significantly less in homogenate incubated with ascorbate in the presence of 100 mM NaCl than in sodium free homogenate. However, it is not clear whether this difference is due to a differential ascorbate effect on agonist vs antagonist receptors, or is simply a manifestation of the intrinsic sodium effect on antagonist binding.

Repeated washing of ascorbate treated homogenates produced no regeneration of ascorbate destroyed SSB. After the first wash no ascorbate could be detected in the supernatants of successive washes when analyzed by HPLC; this would place the ascorbate concentration below 0.01 mM, which is less than that required to produce any significant destruction of SSB. Therefore, ascorbate appears to produce an irreversible loss of opioid SSB.

Scatchard plots showed both loss of [^{3}H]etorphine stereospecific binding sites, and a trend toward decrease in ligand affinity for residual sites in ascorbate treated homogenates. The predominant effect was loss of stereospecific binding sites, with the number of sites being reduced from 14 fmol/mg tissue in control homogenate to 5 fmol/mg tissue incubated for 30 min with 1 mM ascorbate. Homogenate incubated with 20 mM ascorbate lost only 29% of control binding sites, compared to a 64% loss in homogenate incubated with 1 mM ascorbate.

N-Ethylmaleimide is a reagent that destroys SSB by modification of a sulfhydryl group in or near the opioid binding site (14). Both NEM and ascorbate were observed to

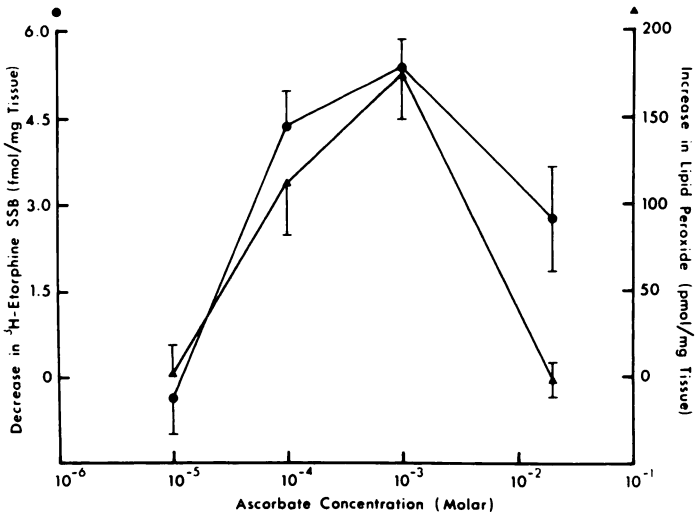

FIG. 6. *Dose-response curves for ascorbate induced lipid peroxide formation and ascorbate destruction of [³H]etorphine stereospecific binding at 22°*

Homogenates were incubated with increasing ascorbate concentrations for one hour at 22°. Following incubation, aliquots from each homogenate were immediately assayed for lipid peroxide formation, while the remainder of each homogenate was washed 2 times and assayed for [³H]etorphine SSB. Values are means ± s.e.m. of four experiments. Decrease in [³H]etorphine SSB in fmol/mg tissue (●) and increase in lipid peroxide formation in pmol/mg tissue (▲) are plotted against the logarithm of molar ascorbate concentration.

produce comparable destruction of SSB. However, while preincubation with levorphanol gave significant protection from NEM destruction of SSB, compared to dextrorphan preincubation, levorphanol gave no protection against ascorbate destruction of SSB. Therefore, ascorbate does not appear to modify any structures directly in, or very near the opioid binding site.

The kinetics of ascorbate inactivation of SSB were studied as a function of temperature and ascorbate concentration. The inactivation was observed to have two components, an initial, rapid phase and a slower phase which followed pseudo-first order kinetics. This finding suggests that ascorbate inactivation of opioid SSB proceeds through more than one mechanism. The slow inactivation was concentration dependent and biphasic at 37°. A 10-fold increase in ascorbate concentration from 0.1 to 1 mM produced a 2.3-fold decrease in reaction half-time. When the ascorbate concentration was increased to 20 mM, the slow inactivation of opioid SSB became negligible. An Arrhenius plot of the rate constants for the slow inactivation of SSB by 1 mM ascorbate produced a value of 20 kcal/mol for the activation energy of the slow inactivation process, a value generally associated with chemical reactions having half-times on the order of minutes to hours (15).

The pH dependence observed for ascorbate inactivation of opioid SSB suggests that an enzyme or other protein containing titratable residues may play some part in the ascorbate inactivation of stereospecific binding. Therefore, the effects of inhibitors of various oxygen and/or energy-utilizing enzymes on ascorbate destruction of SSB were studied. All inhibitors tested were found to be without effect on ascorbate destruction of SSB. If an enzyme is involved in ascorbate induced destruction of SSB, it is not likely to be a cyclo-oxygenase or mixed function oxidase.

Ascorbic acid is commonly used as a biological reducing agent, and chemical reduction was explored as a possible mechanism for ascorbate destruction of SSB. The ascorbic acid/dehydroascorbic acid redox system has an oxidation-reduction potential (E_0') of 0.06 volt at pH 7.0 (16), and other chemical and biochemical reducing agents were selected to produce a range of

TABLE 6

Effects of polar lipids on [^{3}H]etorphine stereospecific binding and ascorbate induced destruction of stereospecific binding in guinea pig brain homogenate

One percent guinea pig brain homogenates containing polar lipids (50 μg/ml) or polyunsaturated fatty acids (5 μg/ml) were incubated in the presence (+Ascorbate) or absence (−Ascorbate) of 1 mM ascorbate for 15 min at 37°, then washed free of ascorbate and excess lipid by two rounds of centrifugation, resuspended to 1 (w/v)% in 100 mM Tris-HCl buffer, and assayed for stereospecific binding with 2.5 nM [^{3}H]etorphine.

Values represent means ± s.e.m. of (*n*) experiments.

Lipid	Percent of Control Stereospecific Binding	
	(−Ascorbate)	(+Ascorbate)
Control homogenate	100 ± 4 (8)	18 ± 2 (8)
Phosphatidyl choline	107 ± 5 (3)	12 ± 6 (3)
Phosphatidyl ethanolamine	110 ± 11 (3)	8 ± 4 (3)
Phosphatidyl inositol	111 ± 18 (3)	20 ± 2 (3)
Phosphatidyl serine	102 ± 8 (8)	46 ± 2 (8)
Cerebroside sulfates	105 ± 9 (3)	21 ± 5 (3)
Cholesterol	96 ± 11 (3)	13 ± 3 (3)
Sphingomyelin	111 ± 13 (3)	22 ± 3 (3)
Arachidonic acid	89 ± 7 (3)	5 + 1 (3)
Docosahexaenoic acid	88 ± 8 (3)	8 ± 3 (3)

redox potentials encompassing this value. Only ascorbic acid produced any significant loss of SSB, ruling out chemical reduction as a possible mechanism for destruction of SSB.

Structure-activity-relationship studies were carried out with analogues of L-ascorbic acid, the structures of which are shown in Figure 4. Each analogue was tested alone for intrinsic effects on opioid SSB, and in combination with 1 mM ascorbate to determine if it antagonized ascorbate destruction of SSB. The stereoisomer, D-isoascorbic acid (II), produced SSB destruction identical to that produced by ascorbic acid. Dehydroascorbic acid (III) produced a small amount of destruction of SSB by itself, but also provided significant protection against ascorbate destruction of SSB. All other analogues tested neither destroyed SSB, nor protected against ascorbate destruction of SSB. Since analogues IV, VI, VII and VIII, in particular, have structures very similar to ascorbic acid, it seems unlikely that there is a specific receptor to which ascorbate binds, initiating a sequence of biochemical events leading to destruction of opioid SSB. Dehydroascorbic acid did produce significant inhibition of SSB destruction, but this compound is an intimate component of the ascorbate redox system, and very possibly may act as a chemical scavenger for some reactive ascorbate intermediate involved in the destruction of opioid SSB.

Ascorbic acid catalyzes the utilization of molecular oxygen in chemical and biochemical reactions (17–19), and results of experiments designed to determine if oxygen was necessary for ascorbate destruction of SSB are presented in Figure 5. These results clearly prove that molecular oxygen is necessary for ascorbate induced destruction of opioid SSB. Ascorbic acid has been known for years to catalyze the peroxidation of biomembrane lipids, and this reaction can

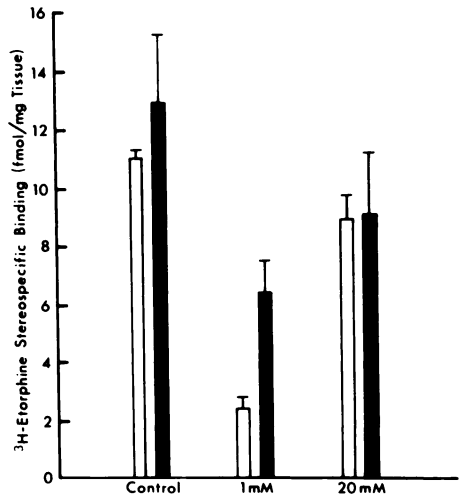

FIG. 7. *Comparison of phosphatidyl serine protection of 2.5 μM [^{3}H]etorphine stereospecific binding against destruction by 1 mM and 20 mM ascorbate*

Bar values are means ± s.e.m. of three experiments. Open bars represent control homogenate incubated with or without ascorbate for 15 min at 37°, and solid bars represent homogenate containing 50 μg/ml phosphatidyl serine, incubated with or without ascorbate for 15 min at 37°. Control: homogenate containing no ascorbate; 1 mM: homogenate incubated with 1 mM ascorbate; 20 mM: homogenate incubated with 20 mM ascorbate.

be inhibited by chelating agents such as EDTA and EGTA, specific antioxidants such as PPDA, and the metal ions, Co^{+2} and Mn^{+2} (20–25). Therefore, these agents, as well as several additional metal ions, were tested both for their effects on ascorbate induced lipid peroxidation and destruction of SSB. From the results presented in Table 5, it is immediately apparent that all of the reagents previously known to inhibit ascorbate induced lipid peroxide formation were also potent inhibitors of ascorbate destruction of opioid SSB. In addition, it was demonstrated for the first time that the metal ions, Cr^{+2} and La^{+3}, also inhibit both lipid peroxidation and ascorbate destruction of SSB. Those metal ions which had no effect on lipid peroxide formation, such as Ca^{+2} and Mg^{+2}, did not prevent ascorbate destruction of stereospecific binding.

Dose-response curves for ascorbate induced lipid peroxidation and destruction of opioid SSB were almost identical up to 1 mM ascorbate, at which concentration both maximum lipid peroxidation and destruction of SSB occur. With 20 mM ascorbate, however, lipid peroxide formation is completely inhibited, while some destruction of SSB still occurs. These observations are consistent with the hypothesis that ascorbate destruction of opioid SSB occurs through two separate mechanisms, and that one of the two mechanisms involves ascorbate catalyzed lipid peroxidation.

Washing of treated homogenate removes lipid peroxide but does not restore SSB, eliminating blockage of the receptor binding site by lipid peroxide as the mechanism for loss of SSB. While destruction of SSB resulting from some other indirect consequence of peroxidation of lipids unassociated with the opioid receptor cannot be ruled out, a more likely hypothesis involves peroxidation of lipid which is intimately associated with the opioid receptor. An ascorbate catalyzed peroxide intermediate is postulated to attack double bonds in the unsaturated fatty acid components of a polar lipid which is essential in maintaining structural integrity of the binding site of the opioid receptor(s), but not in close enough proximity to the binding site to be protected with an opiate ligand. The peroxidation of the fatty acid side chains of the polar lipid presumably causes perturbations in the conformation of the binding site, which lead to loss of SSB. This hypothesis may also account for the reduction in opioid SSB observed by Pasternak *et al.* in rat brain homogenates incubated with Fe^{+2} (26), since this ion is also known to catalyze lipid peroxide formation (23).

Experiments were performed in which homogenates were preincubated with various biologically important lipids to determine if any of these lipids could protect against ascorbate induced destruction of SSB. Although none of the lipids produced any significant change in opioid SSB when incubated with brain homogenate, there was a tendency toward increased SSB above control levels following incubation with most of the lipids tested. These results are not consistent with the work of Abood and Takeda, in which phosphatidyl serine and sulfatides increased SSB by 30% and 17%, respectively, over control binding (11). However, results of these two studies are not directly comparable, since homogenate preparations were washed prior to assay for SSB in the present study. Of the lipids tested, phosphatidyl serine alone gave significant protection from ascorbate destruction of SSB by 1 mM ascorbate. On a molar basis, the concentration of phosphatidyl serine needed for protection is 10–20 times less than the ascorbate concentration employed. Therefore, it is unlikely that phosphatidyl serine protects against SSB destruction by direct interaction with ascorbate. Phosphatidyl serine contains highly unsaturated acyl substituents, which might act as a sink for peroxide intermediates. However, this was found not to be the case, since preincubation with arachidonic acid (20:4 ω6) and docosahexaenoic acid (22:6 ω3) offered no protection against destruction of SSB by 1 mM ascorbate. Attempts at regeneration of SSB by incubation with phosphatidyl serine and other lipids following ascorbate treatment were not successful. Phosphatidyl serine protection against SSB destruction by 1 mM and 20 mM ascorbate was compared. Again, phosphatidyl serine gave good protection against destruc-

tion of SSB by 1 mM ascorbate. However, it gave no protection against destruction of SSB caused by 20 mM ascorbate, providing additional evidence in support of a second mechanism for ascorbate destruction of SSB.

Ascorbic acid was tested in both guinea pig ileum-myenteric plexus and mouse vas deferens opiate bioassays and was found to be completely inert, displaying neither agonist nor antagonist properties. However, SSB in homogenates of guinea pig ileum was subject to almost complete inactivation by ascorbate. These observations lead to speculation concerning the organization of the opioid receptor(s) at the cellular level, and to the relationship of the lipid component (possibly phosphatidyl serine) to the receptor binding site. Recently, evidence was presented that phosphatidyl serine is distributed asymmetrically across cell membranes, occurring predominantly on the interior surface of the cell membranes (27, 28). If the lipid component of the opioid receptor binding site were located intracellularly, this might account for the observation that ascorbate destruction of SSB occurs only in homogenates and not in tissue preparations organized at the cellular level. Then it must be reasoned that either the receptor binding site is located on the interior of the cell, or that structural determinants of the receptor binding site extend through the membrane. Since several lines of evidence indicate that the receptor binding site is located on the external surfaces of neural tissue (6, 29, 30), the latter hypothesis is more viable. Transmembranal communication between the receptor binding site and intracellular phospholipid may represent the first step in the translation of binding of an opioid ligand into a biochemical or physiological effect.

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