Ascorbate enhancement of H1 histamine receptor sensitivity coincides with ascorbate oxidation inhibition by histamine receptors

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Ascorbate enhancement of H1 histamine receptor sensitivity coincides with ascorbate oxidation inhibition by histamine receptors. This activity is mediated by ascorbate binding to the extracellular domain of the adrenergic receptor, which also decreases the oxidation rate of ascorbate. Histamine receptors have extracellular agonist or ascorbate binding sites with strong similarities to \( \alpha_1 \) and \( \beta_2 \)-adrenergic activity. This activity is mediated by ascorbate binding to the extracellular domain of the adrenergic receptor, which also decreases the oxidation rate of ascorbate. H1 histamine receptors have extracellular agonist or ascorbate binding sites with strong similarities to \( \alpha_1 \) and \( \beta_2 \)-adrenergic receptors. Physiological concentrations of ascorbate (50 \( \mu \)M) significantly enhanced histamine contractions of rabbit aorta on the lower half of the histamine dose-response curve, increasing contractions of 0.1, 0.2, and 0.3 \( \mu \)M histamine by two- to threefold. Increases in ascorbate concentration significantly enhanced 0.2 \( \mu \)M histamine (5–500 \( \mu \)M ascorbate) and 0.3 \( \mu \)M histamine (15–500 \( \mu \)M ascorbate) in a dose-dependent manner. Histamine does not measurably oxidize over 20 h in oxygenated PSS at 37°C. Thus the ascorbate enhancement is independent of ascorbate’s antioxidant effects. Ascorbate in solution oxidizes rapidly. Transfected histamine receptor membrane suspension with protein concentration at >3.1 \( \mu \)g/ml (56 nM maximum histamine receptor) decreases the oxidation rate of 392 \( \mu \)M ascorbate, and virtually no ascorbate oxidation occurs at >0.0004 mol histamine receptor/mol ascorbate. Histamine receptor membrane had an initial ascorbate oxidation inhibition rate of 0.094 min\(^{-1}\) \( \mu \)g protein\(^{-1}\) ml\(^{-1}\), compared with rates for transfected ANG II membrane (0.055 min\(^{-1}\) \( \mu \)g protein\(^{-1}\) ml\(^{-1}\)), untransfected membrane (0.052 min\(^{-1}\) \( \mu \)g protein\(^{-1}\) ml\(^{-1}\)), creatine kinase (0.0082 min\(^{-1}\) \( \mu \)g protein\(^{-1}\) ml\(^{-1}\)), keyhole limpet hemocyanin (0.00092 min\(^{-1}\) \( \mu \)g protein\(^{-1}\) ml\(^{-1}\)), and osmotically lysed aortic rings (0.00057 min\(^{-1}\) \( \mu \)g wet weight\(^{-1}\) ml\(^{-1}\)). Ascorbate enhancement of seven-transmembrane-spanning membrane receptor activity occurs in both adrenergic and histaminergic receptors. These receptors may play a significant role in maintaining extracellular ascorbate in a reduced state.

Molecular complementarity; vitamin C; seven-transmembrane-spanning membrane receptors

ASCORBATE HAS BEEN SHOWN TO enhance \( \alpha \)-adrenergic contraction in rabbit aortic smooth muscle (2, 11), producing a leftward shift in the dose-response curve to norepinephrine. Ascorbate also significantly prolonged the contractions produced by norepinephrine, independent of the antioxidant effects of ascorbate. Ascorbate also enhances the \( \beta \)-adrenergic relaxation produced by epinephrine in pulmonary tissues (Dillon PF, Root-Bernstein RS, Robinson NE, Abraham WM, Lieder CM, and Berney C, unpublished observations), increasing the depth of relaxation in porcine trachea precontracted with acetylcholine, as well as reducing the fade of epinephrine relaxation in the trachea, thereby prolonging the relaxation of the trachea. The mechanism of this relaxation is an allosteric effect of the direct binding of ascorbate to the extracellular domain of the adrenergic receptor, prolonging the adrenergic effect, while concurrently maintaining the ascorbate concentration by preventing ascorbate oxidation (Dillon et al., unpublished observations).

The adrenergic receptors are a class of the seven-transmembrane-spanning (7TM) membrane receptor proteins, as is the H1 histamine receptor (7, 8). This receptor is responsible for the contractions of large blood vessels, as are the \( \alpha \)-adrenergic receptors. We sought to test the hypothesis that the ascorbate enhancement of the histaminergic as well as the adrenergic 7TM class of receptor occurs. Using the same tissue in which the \( \alpha \)-adrenergic ascorbate enhancement was discovered, we tested the effects of physiological levels of ascorbate over the histamine dose-response curve, and the effect of varying ascorbate concentrations on histamine contractions. The availability of the transfected human H1 histamine receptor membrane (HRM) allowed us to test whether ascorbate binds to the receptor and the degree to which the histamine receptor may prevent the oxidation of ascorbate, compared with other membrane preparations for transfected ANG II receptor membrane (A2M) and for untransfected membrane (UTM), as well as the soluble proteins creatine kinase (CK) and keyhole limpet hemocyanin (KLH) and osmotically lysed aortic rings. Positive results from these experiments led us to compare the agonist and ascorbate binding regions of the H1 receptor and the \( \alpha_{1A} \)-adrenergic and the \( \beta_2 \)-adrenergic receptors. Strong similarities between the receptors in these regions led us to conclude that ascorbate enhancement of this class of receptors may be a common phenomenon. Additional similarities between these receptors and the H2, H3, and H4 receptors increase the probability that these receptors may also have their activity enhanced by ascorbate and related compounds.

METHODS

Tissue solutions. PSS contained the following (in mM): 116 NaCl, 5.4 KCl, 19 NaHCO\(_3\), 1.1 NaH\(_2\)PO\(_4\), 2.5 CaCl\(_2\), 1.2 MgSO\(_4\), and 5.6 glucose. PSS was aerated with 95% O\(_2\)-5% CO\(_2\) to maintain pH 7.4 and warmed to 37°C before addition to tissue baths. Distilled and filtered water with a resistance of 17 MΩ was used for all experiments. Isosmolar high K\(^+\)-PSS was made by reducing the NaCl concentration to 46 mM and increasing KCl to 75.4 mM. Histamine was obtained from Sigma. Ascorbate was obtained from Aldrich. Solutions of ascorbate and histamine were prepared fresh from powder on the day of the experiment as a concentrated, refrigerated stock and serially diluted in PSS for each experiment for 10 min (to allow warming to 37°C) before each contraction. All components were kept warmed to 37°C before addition to tissue baths. Distilled and filtered water with a resistance of 17 MΩ was used for all experiments. Isosmolar high K\(^+\)-PSS was made by reducing the NaCl concentration to 46 mM and increasing KCl to 75.4 mM. Histamine was obtained from Sigma. Ascorbate was obtained from Aldrich. Solutions of ascorbate and histamine were prepared fresh from powder on the day of the experiment as a concentrated, refrigerated stock and serially diluted in PSS for each experiment for 10 min (to allow warming to 37°C) before each contraction. All components were kept warmed to 37°C before addition to tissue baths. Distilled and filtered water with a resistance of 17 MΩ was used for all experiments. Isosmolar high K\(^+\)-PSS was made by reducing the NaCl concentration to 46 mM and increasing KCl to 75.4 mM. Histamine was obtained from Sigma. Ascorbate was obtained from Aldrich. Solutions of ascorbate and histamine were prepared fresh from powder on the day of the experiment as a concentrated, refrigerated stock and serially diluted in PSS for each experiment for 10 min (to allow warming to 37°C) before each contraction. All components were kept

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separate and refrigerated before mixing and addition to the prewarming chambers.

**Tissue procedures.** All rabbits used were kept in university-approved facilities before experimental use. All animal procedures were approved by the Institutional Animal Care and Use Committee of Michigan State University. Adult New Zealand White rabbits of either sex were relaxed with 55 mg/kg im ketamine. After 15 min, the rabbits were anesthetized with 50 mg/kg ip Nembutal. When the rabbits were unresponsive to toe pinch, the abdomen was opened and the abdominal aorta was exposed. The aorta was teased from the vena cava and clamped at both the rostral and caudal ends. The aorta was removed with the use of surgical scissors and placed in 4°C PSS. The aortic clamps were removed to induce death.

The tissues were prepared for mechanical measurements with procedures previously used for tissue rings (1). The aorta was debried of excess connective tissue, flushed of any remaining blood, and placed in fresh PSS. Aortic rings of 3 mm were cut with a single-edge razor blade, and the rings were placed in fresh PSS. The scissor-cut ends were not used. Two stainless steel loops with a flat, straight central section were passed through the lumen of each aortic ring. Upper and lower loops were secured to Plexiglas-stainless steel clamps with stainless steel screws. The lower clamp was attached to a micrometer (Newport) for length adjustment. The upper clamp was connected to a 50-g force transducer (Kulite Semiconductor) with a gold chain. The force transducers were interfaced with an eight-channel Gould signal conditioner and recorder.

The rings were immersed in 20- or 25-mL aerated, jacketed tissue baths (Harvard Apparatus) and maintained at 37°C with a Haake circulator. After rings were mounted, each ring was stretched to 5 g and allowed to stress-relax for 2 h before activation. If stress relaxation reached 0 g, the ring was restrretched to 2 g and allowed to stress-relax until the passive force was stable. The rings had a stretched linear length of 3–4 mm. This procedure leaves the rings near their optimal length for force development.

The tissues were activated with either K⁺ or histamine. Tests of ascorbate alone were also done by use of concentrations up to 500 µM ascorbate and showed no measurable contraction. Individual contractions were generated by replacing PSS in the tissue baths with prewarmed PSS containing the stimulating agent. An initial K⁺ contraction was made on each ring before any histamine contractions. Each contraction lasted at least 10 min, at which time prewarmed PSS was used to wash out the contracting solution. Relaxation to baseline force typically took 10 min. In addition, either a standard nanomolar norepinephrine- or epinephrine-PSS solution was used at both the beginning of a day’s experiments and at the end to ensure that the force generated by the standard was retained throughout the set of experiments. At the conclusion of the experiments, the rings were removed from the baths, blotted, and weighed on a Mettler balance to the nearest 0.1 mg. For the 22 rings in these experiments, the average wet weight was 7.4 mg (SD 0.8). To minimize error that can be introduced by percent comparisons in dose-response curves, the contractions were also normalized to the weight of the ring (g force/mg tissue).

**Capillary electrophoresis measurement of histamine oxidation.**

The histamine oxidation rate was measured by preparing 1.0 mM ascorbate in PSS. The solution was placed in the same baths used for the tissue contractions, including the stainless steel-Plexiglas clips used to hold the tissue. Samples of these solutions were measured by capillary electrophoresis (2, 4). Samples were vacuum injected into an ISCO 3850 electrophorometer for 2 s at 8.6 nL/s into a 100-µm-diameter, 94-cm-length uncoated glass capillary. The samples were exposed to 20 kV (~80 µA) in a carrier buffer of 20 mM sodium borate, pH 9.4, and measured at a window 66 cm from the injection site at 195 nm. The histamine peak was plotted as a function of time. The solution was sampled multiple times on consecutive days, with the tissue baths sealed to prevent evaporation overnight. The change in peak height over ~24 h was measured. Chemical oxidation of histamine would result in a change in peak height and/or retention time in the capillary electrophorometer.

**Protein assay.** All proteins and membrane preparations were made in 20 mM sodium phosphate (pH 7.4). Protein content was measured with a Bio-Rad protein microassay. Samples were added to a cuvette, and the total sodium phosphate volume was added to 400 µL, followed by 100 µL of undiluted Bio-Rad protein assay dye. The samples were read at spectrophotometer absorbance mode of 595 nm. Standard curves of known concentrations of pyruvate kinase were used for comparison with the membrane samples.

**Membrane preparation.** The availability of human HRM (Jena Bioscience, Jena, Germany) allowed experiments of the effect of ascorbate on the receptor. Human recombinant histamine receptor is transfected in SF9 insect cells and supplied as a membrane suspension in 75 mM Tris·HCl, 12.5 mM MgCl₂, and 1 mM EDTA at pH 7.4. A 500-µL sample of HRM was pelleted at 14,500 rpm for 5 min, the supernatant was removed, and the pellet was washed three times with 20 mM sodium phosphate (pH 7.4). The pellet was resuspended in 20 mM sodium phosphate solution and sonicated for 1.5 h. The solution was centrifuged at 800 rpm for 1 min; 250 µL of the supernatant were removed, and the protein content was measured at 0.8 mg/ml in samples used for differential spectroscopy. H1 histamine receptor has a molecular weight of 55.8 kDa (7, 8). This places the upper limit of the histamine receptor concentration at 14.3 µM. This concentration produces the upper limit of measurements of (in mol histamine receptor/mol ascorbate) oxidation inhibition. For the ascorbate oxidation inhibition experiments, samples from a separate batch of HRM had a protein concentration of 0.68 mg/ml.

A2M and UTM were obtained from Novascience Biosciences (Hanover, MD). A2M receptor was transfected into Chinese hamster ovary cell membranes. The A2M suspension is supplied in 50 mM Tris·HCl containing 10% glycerol, 100 mM NaCl, 1 mM MgCl₂, 0.1 BSA, and 0.1 mM bacitracin (pH 7.2). The UTM suspension is supplied in 50 mM Tris, 12 mM MgCl₂, and 2 mM EDTA (pH 7.4). Both suspensions were prepared in the same way as the HRM suspension: pelleted at 14,500 rpm for 5 min, supernatant removed, and pellet washed three times with 20 mM sodium phosphate (pH 7.4). The pellet was resuspended in 20 mM sodium phosphate solution and sonicated for 1.5 h. The A2M suspension was further diluted 10-fold in sodium phosphate. The suspensions used in the ascorbate oxidation inhibition experiments were 0.696 mg/ml for A2M and 0.271 mg/ml for UTM.

Total membrane density was determined by centrifuging 250 µL of the membrane sodium phosphate preparation three times at 14,500 rpm for 5 min, with water replacing the removed sodium phosphate supernatant between centrifugations. The membranes were vortexed after addition of the water and before centrifugation. After the final water addition, the samples were vortexed, and the entire volume was placed in a preweighed weighbott. The water was allowed to evaporate, and the remaining membrane weight was determined. The density was determined by dividing by the initial 250-µL volume.

**Histamine receptor-ascorbate differential spectroscopy.** Ascorbate was prepared as a 10 mM stock in 20 mM sodium phosphate buffer at pH 7.4. Triplicate samples of 255 µL containing 392 ± 1.7 µM ascorbate (94.1 µg protein/ml) and 0.56 µM (31.4 µg/ml), 0.17 µM (9.4 µg/ml), or 56 nM (3.1 µg/ml) histamine receptor were prepared; 200 µL of each sample were added to a 96-well plate. Spectra from 190 to 310 nm were then collected in 1-nm increments during a series of repeated readings over a 1-h period in a Spectramax spectrophotometer. Difference spectra have been used previously to quantitatively study binding of ascorbate (5, 6). This process allowed measurement of the effect of the presence of histamine receptor on ascorbate oxidation. The effect of histamine receptor on the ascorbate oxidation rate can be measured by the disappearance of the ascorbate resonance, with a peak at 266 nm. The ascorbate oxidation rates were...
measured by plotting the logarithm of ascorbate vs. time, with the rate constant determined from the calculated slope. In the present study, experiments demonstrated that histamine receptor concentrations >170 nM virtually stopped ascorbate oxidation up to the ascorbate concentration tested (392 μM).

Lysed aortic ring preparation. Rabbit aortic rings, prepared as in the mechanics experiments, were place in 20 mM sodium phosphate (pH 7.4) for 72 h. The low osmotic strength of this solution (estimated osmolarity of 56 mM at pH 7.4) will rupture the cell membrane, leaving only the cell remains. The effect of the lysed cell remains on ascorbate oxidation was measured by weighing the aortic ring remains and placing them in fresh sodium phosphate at 20, 6.7, 0.67, 0.2, and 0 mg aortic ring/ml in solutions containing 100 μM ascorbate. Samples of these solutions were placed in the spectrophotometer, and the absorbance at 266 nm was measured. The change in the absorbance was used to estimate the change in ascorbate oxidation produced by the aortic remains relative to ascorbate oxidation alone.

Ascorbate oxidation inhibition by membranes and proteins. Samples were prepared with fresh 1 mM ascorbate, 1 mg/ml protein solutions, and the membrane preparations from above. All samples had ± 25 μM ascorbate, differing amounts of protein or membrane, and sodium phosphate added to bring the total volume to 250 μl. All samples were prepared in triplicate and vortexed. Each sample had 200 μl placed into a 96-well quartz plate. Each experiment had 24 total samples, 3 of these with ascorbate and 3 without ascorbate for each protein or membrane amount, with 4 protein or membrane amounts for each experiment. Ascorbate oxidation was measured in a Molecular Devices scanning spectrophotometer, which measures the absorbance from 190 to 310 nm in 1-nm increments. A new scan was initiated every 8 min. This procedure accelerates the oxidation of ascorbate, which had a time constant (τ) of 6.2 min (SD 1.2) (n = 29), in the absence of other compounds. The τ for each sample was calculated by subtracting the average zero ascorbate sample absorbance for each amount of membrane or protein from the samples containing ascorbate, normalizing the samples to the initial absorbance absorbance at 266 nm (the peak of the ascorbate absorbance), converting the normalized values into their natural log, fitting the log values to the least-square minimizing fitting program, and using the best fit to calculate the estimated value at 1/1 of the initial absorbance (which is −1 log unit) for the value of τ. τ0 is the ascorbate oxidation time constant value at 0 added protein or membrane. Added protein or membrane reduces the oxidation rate and increases τ. The value of (1 − τ0/τ) calculates the amount of inhibition of ascorbate oxidation by the protein or membrane, with a value between 0 (no inhibition) and 1 (complete inhibition, no ascorbate oxidation). This relation is rectilinear, going from 0 and approaching 1 asymptotically. The best fit line is calculated with the linear fit of the inverted inhibition as a function of inverted concentration, forced to go through the point (0, 1) in the inverted fit, the point at which an infinite protein concentration produces complete inhibition of ascorbate oxidation. The fitted equation has the form

\[ I = c/(m + c) \]  

(2)

The derivative of this equation (as a function of the concentration) at c = 0 gives the initial rate of ascorbate oxidation inhibition normalized to the ascorbate oxidation alone. The derivation of this equation is

\[ dI/dc = m(1/c + 1)^2 \]  

at c = 0, \( dI/dc = 1/m \); at \( c = \infty \), \( dI/dc = -mc + 1 \). The value at \( c = 0 \) is used for the comparison of inhibition effects. The best fit lines were converted back to absolute oxidation rates from the rates relative to oxidation with ascorbate alone by dividing by the time constant for ascorbate oxidation under these conditions (6.2 min).

Amino acid matching. The amino acid sequences of H1 (P35367), H2 (P25021), H3 (Q9YSN1), and H4 (Q9H3N8) receptors and α1a (P35348) and β2 (P07550) adrenergic receptors were acquired from the Swiss-Prot data base. Amino acid similarities were compared with the use of the LALIGN program, which finds the best local alignments between two amino acid sequences (9).

Statistics. Comparisons between different samples were made with Student’s two-tailed t-test. \( P < 0.05 \) indicated a significant difference. In the mechanics experiments, the comparisons were always made between contractions by the same ring. For these, the t-test used paired comparisons. Comparisons of the control ascorbate oxidation inhibition with the HRM supernatant and with HRM samples used the t-test for unpaired samples with different variances.

RESULTS

Figure 1 shows a chart record of sequential 0.3 μM histamine contractions of rabbit abdominal aortic rings. The solid line beneath the chart record indicates the presence of histamine ±50 μM ascorbate. The histamine contraction in the presence of ascorbate is higher than in the presence of histamine alone. The ascorbate enhancement only lasts during the contractions. In control experiments, ascorbate was added to the rings in concentrations up to 500 μM. Ascorbate alone does not produce any detectable contraction at any concentration tested. The ascorbate enhancement does not carry over to subsequent contractions but is rapidly removed after the ascorbate is washed out.

Figure 2 shows the dose-response curves for histamine in the presence and absence of 50 μM ascorbate. There is a significant increase in the contractions produced by 0.1, 0.2, and 0.3 μM histamine when ascorbate is present. The average effect of 50 μM ascorbate for these three concentrations is a fractional increase of 2.33, 2.84, and 1.81, respectively. There is no significant increase in the contractions produced in the presence and absence of 50 μM ascorbate. There is a significant increase in the contractions produced by 0.1, 0.2, and 0.3 μM histamine when ascorbate is present.
increased contractions with 0.2 μM histamine contractions. Ascorbate at 15–500 μM produces significant increases in 0.3 μM histamine contractions. The increases in ascorbate enhancement at supraphysiological concentrations (>100 μM ascorbate) are in contrast to adrenergic ascorbate enhancement, where the maximum ascorbate effect is produced in the physiological range.

Histamine in PSS at 37°C was measured with capillary electrophoresis over 2.8 h (SD 1.7), producing a relative measure of 1 (SD 0.060); n = 5. The same solution was continuously oxygenated at 37°C until the following day, when after 23.3 h (SD 0.6) the histamine had a measure of 1 (SD 0.059); n = 3. Under these conditions, histamine does not measurably oxidize. Thus the enhancement of histamine contractions by ascorbate cannot be due to the prevention of histamine oxidation.

The oxidation of ascorbate, with an initial concentration of 392 μM, is shown in Fig. 4. The spectra of histamine receptor plus ascorbate show that the contributions in the 260- to 270-nm range are dominated by ascorbate. The highest peak has the highest histamine receptor concentration and thus the least ascorbate oxidation. As the histamine receptor concentration decreases, the peak height also decreases, indicating increased ascorbate oxidation. Figure 4 also indicates the oxidation of ascorbate in the absence of histamine receptor. Figure 4, bottom, shows the difference spectra of ascorbate from 190 to 266 nm, the highest ascorbate absorbance wavelength, at different times in the presence of different maximum histamine receptor concentrations. The steepest line shows the oxidation of ascorbate in the absence of histamine receptor. The top two lines are virtually flat, indicating that there is no net oxidation of ascorbate in the presence of this amount of histamine receptor. The rate constants for oxidation of ascorbate were calculated from this data. Figure 5, bottom, shows the oxidation rates of ascorbate at different histamine receptor concentrations. Above a ratio of 0.0004 mol histamine receptor/mol ascorbate, there is no net oxidation of ascorbate. Because this calculation is based on the assumption that all of the protein in HRM is H1 receptor, the 0.0004 ratio is the maximum ratio needed to prevent oxidation.

Figure 6 shows the inhibition of ascorbate oxidation by the three membrane suspensions: histamine, A2M, and UTM. All have significant ability to inhibit ascorbate oxidation. HRM has the highest initial inhibition of the ascorbate oxidation rate, 0.0179 min⁻¹·μg membrane wt⁻¹·ml⁻¹, compared with 0.0047 for A2M and 0.0042 for UTM. The three preparations have protein concentrations of 5.3 (HRM), 11.5 (A2M), and 12.6 (UTM) μg protein/μg membrane. When the ascorbate oxidation inhibition rates are compared based on their protein concentration, HRM has the highest rate of different histamine receptor concentrations in the presence and absence of ascorbate after 20 min of oxidation. β2- Receptors have been shown to have similar effects on ascorbate oxidation.

Figure 5 shows the calculation of the ascorbate oxidation derived from Fig. 4, in which the initial ascorbate concentration was 392 μM. Figure 5, top, shows the peak heights of the absorbance at 266 nm, the highest ascorbate absorbance wavelength, at different times in the presence of different maximum histamine receptor concentrations. The steepest line shows the oxidation of ascorbate in the absence of histamine receptor. The top two lines are virtually flat, indicating that there is no net oxidation of ascorbate in the presence of this amount of histamine receptor. The rate constants for oxidation of ascorbate were calculated from this data. Figure 5, bottom, shows the oxidation rates of ascorbate at different histamine receptor concentrations. Above a ratio of 0.0004 mol histamine receptor/mol ascorbate, there is no net oxidation of ascorbate. Because this calculation is based on the assumption that all of the protein in HRM is H1 receptor, the 0.0004 ratio is the maximum ratio needed to prevent oxidation.

![Fig. 2. Histamine dose-response curves in the presence and absence of 50 μM ascorbate. Values are means ± SE. Similar to the effect of ascorbate on adrenergic contractions of rabbit aorta, low agonist concentration contractions were significantly enhanced by ascorbate, but there was no increase (or decrease) in the maximum force produced by histamine when ascorbate was present. *Significantly different (P < 0.05).](image)

![Fig. 3. Ascorbate dose-response curves showing the enhancement of 0.2 (○) and 0.3 (●) μM histamine [force (SE)] contractions of rabbit aortic rings. The open symbols are the contraction forces in the absence of ascorbate. In contrast to adrenergic enhancement, which did not show continued enhancement above the normal ascorbate range (40–100 μM), the 500 μM ascorbate + 0.3 mM histamine contraction is significantly greater than the 50 μM ascorbate + 0.3 histamine contraction, and the 500 and 150 μM ascorbate + 0.2 μM histamine contractions are significantly greater than all lower ascorbate + 0.2 histamine contractions. *Significantly different (P < 0.05) compared with control contraction in the absence of ascorbate.](image)
0.094 min⁻¹·μg⁻¹·ml⁻¹, compared with 0.055 for A2M and 0.052 for UTM (Table 1). These are much higher rates than those for the soluble proteins CK and KLH (Table 1).

Figure 7 shows the ascorbate oxidation inhibition of HRM, CK, and KLH vs. their protein concentration. HRM inhibits ascorbate oxidation at a rate that is 10 times higher than CK (0.0082 min⁻¹·μg⁻¹·ml⁻¹) and 100 times higher than KLH (0.00092 min⁻¹·μg⁻¹·ml⁻¹). The rate for KLH is close to that for lysed aortic rings in Fig. 8, the inhibition rate of which (0.00057 min⁻¹·μg⁻¹·ml⁻¹) is normalized to the total tissue weight and not the protein content. The mixed protein content of the lysed aortic rings would reflect the ascorbate oxidation inhibition of all the available proteins in this preparation.

Figure 1 shows that ascorbate has a profound effect on histamine-induced contractions of the aorta. The ascorbate effect is rapid, as it greatly increases the contractions when added with histamine, i.e., within minutes. In the example shown, there is more than a 30% increase in the histamine force within 10 min of histamine exposure, an increase that is below the average of an 81% increase for 0.3 μM histamine,
shown in Fig. 2. The effect is also reversible, as evidenced by the sequential contractions. It is highly improbable that this could be caused by any alteration in protein synthesis because of the rapid time scale. Also, histamine does not oxidize significantly in PSS at 37°C for over 20 h. Thus ascorbate protection of histamine from oxidation cannot be the mechanism of ascorbate enhancement. Given these two findings, the most probable effect of ascorbate is on the histamine receptor.

The force enhancement that occurs at low histamine concentrations echoes a similar effect found on H₁ receptors activation of this tissue (2). The lower histamine concentrations of 0.1, 0.2, and 0.3 M result in increases of 2.33, 2.84, and 1.81 times when used with ascorbate compared with histamine alone. In both cases, high levels of agonists that reach the top of the dose-response curve are not further enhanced by the physiological concentration of ascorbate, with histamine concentrations of 0.5, 1.0, and 2.0 μM having fractional forces with 50 μM ascorbate of 1.14, 0.99, and 1.08, respectively, none of which is significantly different from histamine alone. This is consistent with ascorbate increasing the affinity of the agonist for the receptor but not increasing the maximum effect that the receptor can produce. Unlike the naturally occurring adrenergic agonist, norepinephrine, supra-

![Fig. 6. Inhibition of ascorbate oxidation by HR, ANG II receptor, and untransfected (UT) membrane suspensions. The symbols are means (SD) for 3 time constants at each amount of membrane. Inset: inverse plot of 1/inhibition vs. 1/membrane weight. Tau, Time constant. The best fit linearizations of the inverse plots were used to generate the rectilinear curved lines in the larger graph. HR membrane showed the greatest ability of the 3 to inhibit ascorbate oxidation, but all 3 had greater inhibition than any soluble proteins tested.](image1)

![Fig. 7. Inhibition of ascorbate oxidation by HR membrane, creatine kinase, and keyhole limpet hemocyanin. The symbols are means (SD) for 3 time constants at each protein concentration. The rectilinear lines are generated by inverse plots (not shown) of 1/inhibition vs. 1/protein concentration. The initial slopes of the inhibition lines show that HR membrane has a 10-fold greater inhibition than creatine kinase and a 100-fold greater inhibition than keyhole limpet hemocyanin.](image2)

![Fig. 8. Inhibition of ascorbate oxidation by lysed aortic rings. The plot is similar to that in Fig. 7, except that the abscissa is in mg tissue wet wt, rather than in μg protein/ml. The initial slope, although far slower than HR membrane, is similar to keyhole limpet hemocyanin. The weight of seven-transmembrane-spanning membrane receptors in the lysed rings would be only a small fraction of the total weight, most of which would be structural and contractile proteins. Tissue, even dead tissue, can contribute to the maintenance of the reduced state of ascorbate.](image3)

<table>
<thead>
<tr>
<th>Oxidation Inhibitor</th>
<th>Initial Inhibition Rate, minμg⁻¹·ml⁻¹</th>
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</thead>
<tbody>
<tr>
<td>Histamine receptor membrane</td>
<td>0.094</td>
</tr>
<tr>
<td>ANG II membrane</td>
<td>0.055</td>
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<tr>
<td>Untransfected membrane</td>
<td>0.052</td>
</tr>
<tr>
<td>Creatine kinase</td>
<td>0.0082</td>
</tr>
<tr>
<td>Keyhole limpet hemocyanin</td>
<td>0.00092</td>
</tr>
<tr>
<td>Lysed aortic rings</td>
<td>0.00057</td>
</tr>
</tbody>
</table>

Initial inhibition rate is measured in micrograms of protein for all values, except for the aorta, which is in micrograms wet weight.
complex function of the ascorbate, agonist, and receptor interactions. At this time, there can be no a priori determination of an untested combination. Because ascorbate enhancement of phenylpropanolamine and ephedrine could have played a role in the pathological effects produced by these two compounds, further experiments on ascorbate’s possible in vivo alteration of histamine, and antihistamine, functions are required. Antihistamines that bind to the agonist site on the receptor may also be subject to ascorbate enhancement.

Figure 9 shows the amino acid sequences of the H1 histamine and α1a- and β2-adrenergic receptors. These sequences have been shown to have adrenergic agonist and ascorbate binding sites (2, 11, Dillon et al., unpublished observations). The histamine receptor has a strong similarity to both adrenergic regions. The similarity of the effect of ascorbate on both histamine and adrenergic contractions, and of the receptors on ascorbate oxidation, is striking.

The agonist-ascorbate amino acid homologies of all the histamine receptors (H1, H2, H3, and H4) are similar to the same regions on the adrenergic receptors (Fig. 9). The H2, H3, and H4 receptors may show ascorbate enhancement similar to that shown by the H1 receptor. Because of the complex nature of the body’s histamines, e.g., constriction of large vessels and dilation of small vessels, again it is not possible to foretell how the antihistamine function will be altered by ascorbate. In any case, this represents an area of significant clinical possibilities.

The spectra of ascorbate and the H1-receptor-transfected membrane show strong similarities to those of ascorbate and the β2-adrenergic receptor membrane (Dillon et al., unpublished observations), with strong absorbance below 210 nm but little absorbance above this frequency. Both significantly decrease the rate at which ascorbate oxidation takes place. The oxidized product of ascorbate, dehydroascorbate, does not absorb in the 230- to 290-nm range. Thus, even in the presence of H1 receptor, unoxidized ascorbate dominates this region. The rate of ascorbate oxidation is illustrated in Fig. 4, in which the absorbance centered at 266 nm shows a progressive decrease with time in the absence of H1 receptor. Figure 5, top, shows examples of how different concentrations of H1 receptor can affect ascorbate oxidation. The top two lines have almost no ascorbate oxidation, with the difference in heights because of the higher H1 receptor content. As shown in Fig. 5, bottom, H1 histamine receptor and 56 nM H1 receptor have absorbance lower than the other histamine receptor amounts. No H1 receptor present and 56 nM H1 receptor cannot maintain the ascorbate, and it oxidizes. Qualitatively, the β2-adrenergic receptor has been shown similarly to prevent ascorbate oxidation (Dillon et al., unpublished observations), but this is the first quantitative demonstration of the inhibition of ascorbate oxidation.

The maximum rate of ascorbate oxidation under these conditions is shown in Fig. 5. The rate constant for this oxidation was calculated from the disappearance of the ascorbate peak shown in Fig. 4, bottom. If enough H1 receptor is present, there is virtually no oxidation of ascorbate. The amount of H1 receptor needed to prevent oxidation is shown to be far below an equal mole-for-mole ratio, with no significant oxidation occurring at 0.0004 mol H1 receptor/mol ascorbate. This ratio is the maximum ratio, since the calculation assumes that all of the protein in the HRM is histamine receptor. Therefore, the prevention of oxidation cannot be due to ascorbate directly binding to a single specific site on the H1 receptor, as there is far too little H1 receptor available to bind all of the ascorbate. The peak ascorbate in Fig. 4, bottom, with no H1 receptor, is smaller than the peak ascorbate with H1 receptor in Fig. 4, top. Time 0 reflects the time at the start of the experiment in the spectrophotometer. The lower ascorbate in the absence of H1 receptor reflects the time taken for sample preparation, during which some ascorbate oxidation presumably occurs.

Figures 5, 6, and 7 and Table 1 consider the inhibition of ascorbate oxidation by other membrane preparations, soluble proteins, and lysed aortic rings. Figure 5 compares the HRM inhibition of oxidation to the inhibition by A2M and UTM. The A2M inhibition is particularly interesting because ascorbate does not enhance ANG II contraction of the rabbit aorta (2). The UTM preparation, although not transfected with a particular receptor, still presumably has a number of 7TM other receptors on its surface, but with lower protein content than either HRM or A2M preparations. Despite these differences, all three show high levels of inhibition of ascorbate oxidation, with HRM (0.094 min⁻¹·μg⁻¹·ml⁻¹) having less than twice the initial inhibition rate compared with A2M (0.055 min⁻¹·μg⁻¹·ml⁻¹) and UTM (0.052 min⁻¹·μg⁻¹·ml⁻¹) when normalized to protein content. Even assuming the higher protein content in HRM (592 μg/ml) compared with UTM (271 μg/ml) is entirely H1 receptor (remembering these are from different cell lines), the ascorbate oxidation by the H1 receptor is only about twice that of other membrane proteins, suggesting that some of the re-
ceptors in UTM may also have a high degree of ascorbate binding activity. The membrane ascorbate oxidation inhibition is in sharp contrast to that of soluble proteins. Although only a few proteins have been tested, in Fig. 7 and Table 1, ascorbate oxidation inhibition is 10 (CK) to 100 (KLH) times less than that for HRM. Because high enough concentrations of both of these proteins can radically reduce ascorbate oxidation, binding of ascorbate to these proteins presumably does occur. The difference between CK and KLH indicates that ascorbate does not have its binding to the peptide bond, since that would be similar in both proteins, as it would also be in the HRM. There may be specific amino acid sequences that bind to ascorbate that produce a range of ascorbate binding and thus a range of ascorbate oxidation inhibition. The oxidation inhibition by the lysed aortic rings (Fig. 8, Table 1) is over 150 times less than the HRM, but the lysed rings are normalized to tissue wet weight and not protein content. If the membrane proteins that so effectively reduce ascorbate oxidation in the membrane preparations comprise 1/150 of the remaining lysed aortic ring proteins, not an unreasonable estimation, the rate per membrane protein would be similar to that of the membrane preparations. In their review of regeneration of vitamin C, Wells and Jung (11) conjecture that the binding of dehydroascorbate to proteins might be universal. They further note that, in addition to dehydroascorbate reduction to ascorbate by glutathione, this process also occurs enzymatically in proteins with sulfur-rich regions. The presence of cysteine and methionine in all of the amino acid sequences in Fig. 9 makes this a possibility for these receptors.

In summary, we have found that histamine activity in aortic rings can be significantly enhanced by ascorbate. Histamine and other membrane receptors in turn can prevent the oxidation of ascorbate, maintaining it in the reduced state in the extracellular environment. The prevention of ascorbate oxidation in turn will allow ascorbate to enhance histaminergic activity.

Combined with previous findings that ascorbate can enhance adrenergic activity, ascorbate interaction with other 7TM systems should be considered.

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GRANTS
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REFERENCES