Inhibition of Stimulated Ascorbic Acid and Luteinizing Hormone-Releasing Hormone Release by Nitric Oxide Synthase or Guanyl Cyclase Inhibitors

SHARADA KARANTH, WEN H. YU, CLAUDIO A MASTRONARDI, AND SAMUEL M. MCCANN[1]

Pennington Biomedical Research Center, Louisiana State University, Baton Rouge, Louisiana 70808–4124

Ascorbic acid (AA), an antioxidant, is present in high concentrations in the hypothalamus. Previously, we have shown that AA inhibited stimulated release of luteinizing hormone-releasing hormone (LHRH) from medial basal hypothalami *in vitro*. We have also demonstrated that cell membrane depolarization by high [K^+] media-induced AA release that is blocked by N^G-monomethyl-L-arginine, a competitive inhibitor of nitric oxide synthase (NOS), indicating that the release process is mediated by NO. The release of LHRH is also mediated by NO. We hypothesized that AA is a co-transmitter released with classical transmitters from synaptic vesicles that acts to reduce chemically the NO formed, thereby providing feed-forward inhibitory control over LHRH release. Because NO acts by activating guanylyl cyclase (GC) resulting in production of cGMP, in the present investigation we studied the effects of an NOS inhibitor LY 83583 and GC inhibitor, O.D.Q. to further characterize the role of NO in high [K^+]-induced AA and LHRH release. Medial basal hypothalami were incubated in 0.5 ml of Krebs-Ringer Bicarbonate buffer or medium containing increased potassium [K^+ = 56 m*M*] for 1 hr or combinations of high [K^+] + LY 83583 or O.D.Q. for 1 hr. AA and LHRH released into the incubation medium were measured by high-pressure liquid chromatography and radioimmunoassay, respectively. Cell membrane depolarization with high [K^+] produced a significant increase in both AA and LHRH release. A combination of high [K^+] + LY 83583 or high [K^+] + O.D.Q. decreased basal AA and completely blocked high [K^+]-induced AA and LHRH release. As in the case of high [K^+], LHRH release induced by the excitatory amino acid *N*-methyl-D-aspartic acid (NMDA) was blocked by both the inhibitors. NMDA alone failed to alter AA release, but the combined presence of NMDA and the inhibitors totally blocked AA release. Because LY 83583 and O.D.Q. were shown to inhibit NOS and soluble GC, respectively, the data demonstrate that basal and high [K^+]-induced AA and high [K^+] and NMDA-stimulated LHRH release were mediated by NO by its activation of GC and consequent generation of cGMP.
Exp Biol Med 229:72–79, 2004

Key words: NMDA; high [K^+] media; LY 83583 (NOS inhibitor); O.D.Q. (GC inhibitor)

Ascorbic acid (AA) plays a major role as an antioxidant and neuromodulator in the brain (1). It enters the central nervous system (CNS) by an active transport mechanism at the choroid plexus (2). It diffuses from cerebrospinal fluid (CSF) to brain extracellular fluid and is taken up by the brain. AA is concentrated several-fold in both CSF and the brain, resulting in higher brain levels of AA than in the CSF, and these greatly exceed those of plasma (3). The brain maintains a high concentration of AA independent of its ingestion (4–7). AA is transported into CSF and neurons by sodium-dependent saturable uptake systems (8). Two types of transporters, a low and high affinity, have been identified in human neutrophils (9). These transporters pump AA from plasma against a much higher gradient so that the intracellular AA remains higher than plasma (9). Two isoforms of the sodium-dependent vitamin C transporters, SVCT1 and SVCT2, have been cloned (10, 11). SVCT1 is mainly located in the epithelial tissues, whereas SVCT2 is confined to special tissues, such as the brain, eye, anterior and intermediate lobe of the pituitary, pancreas, and adrenal cortex and is primarily responsible for AA accumulation in the brain and CSF (10, 11). AA is present in high concentrations in the hypothalamus, but its role in the modulation of hypothalamic hormone release is incompletely understood (12–14).

Nitric oxide (NO) is a diffusible, gaseous neurotransmitter that plays an important role in multiple biological functions, such as neurotransmission, immunological defense, vasodilation, and endocrine signaling (15–18). It is synthesized from the substrate L-arginine via the catalytic

This work was supported by the National Institutes of Health Grant MH51853.
[1] To whom requests for reprints should be addressed at Pennington Biomedical Research Center, Louisiana State University, 6400 Perkins Road, Baton Rouge, LA 70808–4124. E-mail: mccannsm@pbrc.edu

Received July 25, 2003.
Accepted September 9, 2003.

1535-3702/04/2291-0001$15.00

action of a calcium/calmodulin dependent enzyme, NO synthase (NOS) (19–23). NO stimulates soluble guanylyl cyclase (GC) that catalyzes the conversion of guanosine triphosphate (GTP) to the secondary messenger cGMP (16, 20). The cGMP by activating protein kinase G participates in the exocytosis of secretory granules. This chain of events represents a widespread signal transduction mechanism that is involved in a variety of biological processes (22, 24). Impairment of the NO/cGMP pathway has been implicated in a variety of pathological diseases such as diabetes, hypertension, coronary heart disease, and chronic heart failure (25–29). The beneficial effect of AA on several cardiovascular diseases is well established (30).

Luteinizing hormone (LH)-releasing hormone (LHRH) release is controlled by release of NO from NOergic interneurons that release the soluble gas in juxtaposition to the LHRH terminals (31, 32). In the terminals, NO activates GC leading to an increase in cGMP, cyclo-oxygenase, and lipoxygenase that results in increased concentrations of prostaglandin E_2 and leukotrienes, respectively (33–36). All of these participate in causing extrusion of the LHRH secretory granules into the hypophyseal portal vessels for delivery of LHRH to the pituitary gland that results in the release of LH. Sodium nitroprusside (NP), a spontaneous producer of NO, also induces LHRH release. N-methyl-D-aspartic acid (NMDA), an excitatory amino acid, stimulates LHRH release by activating NOS (32, 37–41). The stimulation is blocked by NOS inhibitors such as N^G-monomethyl-L-arginine (NMMA), suggesting that NO plays an important role in NMDA and NP-induced LHRH release (32, 42, 43). Recently we reported that AA acts as an inhibitory transmitter in the hypothalamus (37). It inhibits NMDA and NP-stimulated LHRH release presumably by scavenging NO (37). We have also shown the converse of this effect, namely that incubation of medial basal hypothalamus (MBH) with either NP or NMDA stimulates LHRH release but decreases the concentration of AA in the media probably by NO-mediated oxidation of AA. Collectively these data are suggestive of a major role for AA in the NO/cGMP signaling pathway.

The availability of specific inhibitors of NOS and GC has increased our understanding of the NO/cGMP signaling pathway (44–46). The compound LY 83583 (6-anilino-5, 8-quinolinedione) has been shown to reduce cGMP levels in various tissues without altering cAMP (44, 45). It has been shown to reduce cGMP by inhibiting NOS, thereby reducing NO synthesis resulting in failure to activate GC that converts GTP to cGMP. Recently a selective inhibitor of the soluble form of the GC has been identified (46, 47). This new compound, 1H-[1,2,4] oxidizable [4,3-a] quinoxalin-1-one (O.D.Q.), has been shown to inhibit selectively soluble GC both *in vivo* and *in vitro* (46, 47).

Recently AA has been shown to inhibit purified GC (48). This action of AA may be another mechanism by which it inhibited LHRH stimulation as well as by scavenging NO (37). Therefore, it is of interest to examine further the role of NOS and GC inhibitors in the action of AA. The present investigation was designed to elucidate the effect LY 83583 and O.D.Q. on high potassium-induced AA and LHRH release from incubated MBH. The effect of LY 83583 and O.D.Q. on AA and LHRH release has also been studied in the presence of NMDA, a known stimulator of LHRH release.

Materials and Methods

Adult male rats of the Sprague Dailey strain (Holtzman, Madison, WI, 200–250 g) were housed two per cage under controlled conditions of temperature (23–25°C) and lighting (on 0500–1700 hr). The animals had free access to a pellet diet consisting of Purina Lab rat chow 5001 and tap water.

Chemicals. Sodium ascorbate, NMMA, NMDA, bacitracin, and O.D.Q. were purchased from Sigma (St. Louis, MO, USA). LY 83583 was obtained from Research Biochemicals International (NA tick, MA).

***In Vitro* Studies.** *Incubation of MBH.* Animals were sacrificed by decapitation, and the brain was exposed by a dorsal incision. MBH were dissected by vertical cuts along the lateral hypothalamic sulci, posterior edge of the optic chiasma, and the anterior edge of the mammillary bodies. A horizontal cut 2 mm from the base separated the island. MBS (8–12 mg) were incubated *in vitro* as previously reported (37). In brief, one MBH/tube was placed in 0.5 ml of Krebs-Ringer bicarbonate (KRB) (pH 7.4) buffer supplemented with 20 μM bacitracin in an atmosphere of 95% O_2 and 5% CO_2 in a Dubroff shaker (50 cycles/min) for a period of 60 mins. Following this preincubation, the tissues were incubated with 0.5 ml KRB or KRB + $[K^+]$ or a combination of $[K^+]$ + LY 83583 or $[K^+]$ + O.D.Q. for 1 hr. Experiments were also performed to study the effect of NMDA or a combination of NMDA + LY 83583 or NMDA + O.D.Q. Constant ionic strength was maintained by equivalent reduction of sodium ion whenever, KRB + $[K^+]$ was used. At the end of this procedure, medium was aspirated and medium and tissues were stored at −80°C. AA and LHRH were analyzed by high-pressure liquid chromatography (HPLC) and radioimmunoassay, respectively.

Chromatography. Isocratic analysis were carried out with Beckman system gold HPLC equipped with 126 module and diode array detector 168 operating at 254 nm at a sensitivity of 0.016 a.u.f.s (Beckman Instruments Inc, Fullerton, CA). The separation was carried out on a μBond pack Beckman ultra sphere C18 column (average particle size 5 μm, 25 cm × 4.6 mm). The mobile phase was a buffer consisting of 0.1 *M* sodium dihydrogen phosphate ($Na\ H_2\ PO_4$) and 0.2 m*M* Na_2 EDTA adjusted to pH 3.1 with orthophosphoric acid. The buffer was filtered through a 0.45-μm membrane filter (Gelman Sciences, MI) and degassed prior to use. The column was maintained at room temperature and the mobile phase was used at a constant flow rate of 1.0 ml/min.

Preparation of Standard. A sample buffer consisting of 5 m*M* each of metaphosphoric acid and Na_2EDTA was

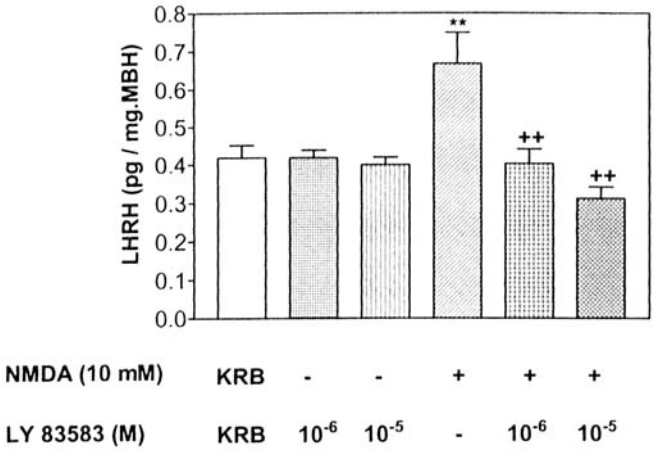

Figure 1. Influence of NMDA or combination of NMDA + LY 83583 on LHRH release after 1 hr of incubation. In this and subsequent figures, the results are the mean ± SEM. Number of tissues for each group was 8. **, $P < 0.01$ versus KRB. ++, $P < 0.01$ versus the group treated with NMDA.

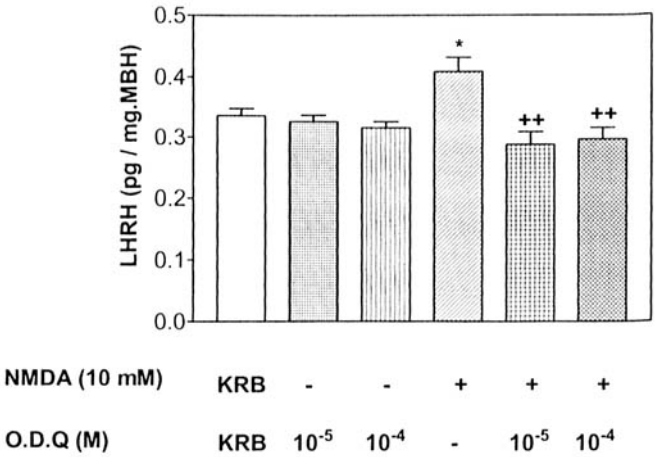

Figure 2. Effect of NMDA or combination of NMDA + O.D.Q. on LHRH release. *, $P < 0.05$ versus KRB. ++, $P < 0.01$ versus the group treated with NMDA.

prepared in HPLC-grade water (V.W.R Scientific Products, TX) and was used for preparing AA standards and MBH homogenates. The sample buffer was previously shown to stabilize AA solution for 3–4 hrs, and all the estimations were completed within this time (49). A standard curve for AA was prepared from a stock solution of 1 mg/ml and was linear from 487.5 to 7800 ng. AA in standards, incubation medium, and homogenates were measured using 507 ASE auto sampler (Beckman), and samples were used at a volume of 30 µl. Each sample (unknown) was passed through syringe filters (Gelman Sciences) before placing in the vial for counting. A standard calibration plot was obtained for AA concentrations (micrograms per milliliter) versus peak area (numerical units on 126 module). Multiple plots for standard curve were constructed using freshly prepared samples on different days.

LHRH Assay. A highly specific antibody to LHRH was provided by Dr. A Barnes (University of Texas Southwestern Medical Center, Dallas). The minimal detectable LHRH was 0.2 pg/tube, and the curve was linear up to 100 ng/tube.

Statistics. Results were analyzed by one-way analysis of variance or paired Student's t tests wherever applicable and $P < 0.05$ was considered significant.

Results

Effect of NMDA or a Combination of NMDA + LY 83583 on LHRH Release. Incubation of MBH with two different doses of LY 83583 (10^{-6} and 10^{-5} M), an inhibitor of NOS, failed to alter basal LHRH release (Fig. 1). NMDA significantly stimulated LHRH release, and NMDA-induced LHRH release was markedly decreased in the presence of both doses of LY 83583.

Effect of NMDA or a Combination of NMDA + O.D.Q. on LHRH Release. Incubation of MBH with two different doses of O.D.Q. (10^{-5} and 10^{-4} M), a specific inhibitor of the enzyme soluble GC, for 1 hr did not alter basal LHRH release (Fig. 2). NMDA alone significantly stimulated LHRH release. A combination of NMDA + either dose of O.D.Q. significantly reduced LHRH release and the effect was not dose dependent.

Effect of High [K^+] or High [K^+] + LY 83583 on LHRH Release. High [K^+] stimulated LHRH release and incubation of MBH with two different concentrations of LY 83583 (10^{-6} and 10^{-5} M) failed to alter basal LHRH release (Fig. 3). A combination of high [K^+] plus LY 83583 significantly suppressed LHRH release, and the suppression induced by both doses was comparable.

Effect of High [K^+] or a Combination of High [K^+] + O.D.Q. on LHRH Release. Incubation of MBH with two different doses of O.D.Q. failed to alter basal LHRH release (Fig. 4). High [K^+] stimulated LHRH release, and both doses of O.D.Q. markedly suppressed LHRH release, but the effect was not dose dependent.

Effect of NMDA or a Combination of NMDA + LY 83583 on AA Release. Both doses of LY 83583 decreased basal AA release, and the effect was dose dependent (Fig. 5). At the higher concentration, AA release was nearly completely blocked and was significantly lower than the release with the lower concentration of LY 83583. NMDA failed to alter AA release. A combination of NMDA and both doses of LY 83583 suppressed AA release, and the effect was dose dependent.

Effect of NMDA or a Combination of NMDA + O.D.Q. on AA Release. Incubation of MBH with the lower dose of O.D.Q. failed to alter basal AA concentration, but the higher dose significantly suppressed AA release (Fig. 6). NMDA failed to alter AA release, but a combination of NMDA and both doses of O.D.Q. significantly suppressed AA release with a greater suppression achieved by the higher dose.

Effect of High [K^+] or High [K^+] + LY 83583 on AA Release. Incubation of MBH with two different

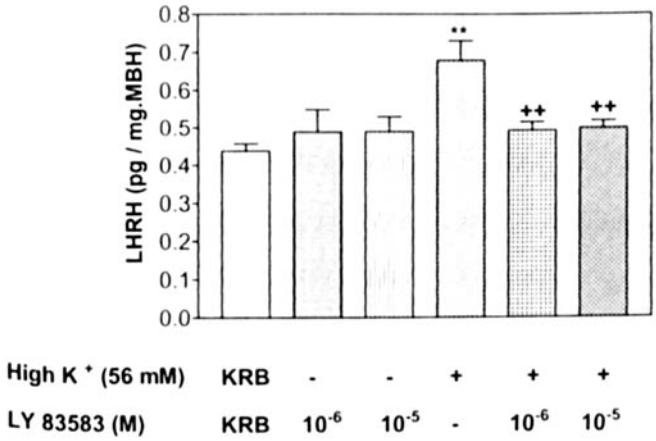

Figure 3. Influence of high [K^+] or combination of high [K^+] + LY 83583 on LHRH release. **, $P < 0.01$ versus KRB. ++, $P < 0.01$ versus the group treated with high [K^+].

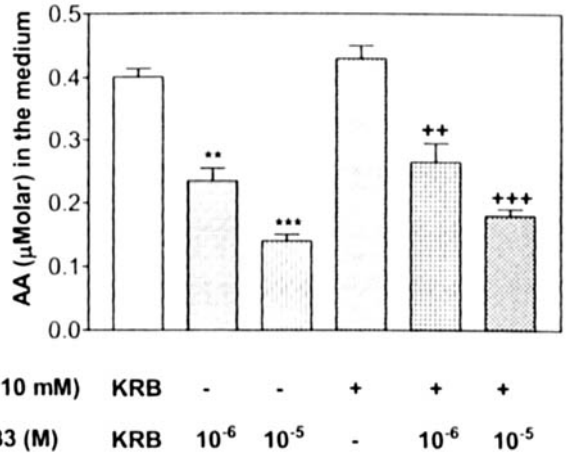

Figure 5. Influence of NMDA or combination of NMDA + LY 83583 on AA release into the medium. **, $P < 0.01$ or ***, $P < 0.001$ versus KRB. ++, $P < 0.01$ or +++, $P < 0.001$ versus the group incubated with NMDA.

concentrations of LY 83583 (10^{-6} and 10^{-5} *M*) resulted in a significant decrease in basal AA release, and the effect was dose dependent (Fig. 7). At the higher concentration, AA release was completely blocked and was significantly lower than the basal release. High [K^+] stimulated AA release that was significantly suppressed by both doses of LY 83583. A combination of high [K^+] + the higher concentration of LY 83583 decreased AA significantly below the basal release.

Effect of High [K^+] or a Combination of High [K^+] + O.D.Q. on AA Release. The lower dose of O.D.Q. failed to modify, and the higher dose significantly lowered basal AA release (Fig. 8). High [K^+] stimulated AA release. Both doses of O.D.Q. significantly reduced AA release induced by high [K^+], but the effect was not dose dependent.

Discussion

NO functions as an important neurotransmitter in the brain (15–17). Substantial evidence indicates that glutamate activates NOS in the brain and in particular in the MBH (41, 50, 51). The close proximity of GnRH neurons to the anatomical localization of NO neurons in the hypothalamus further supports an important role for NO in the regulation of GnRH secretion. This view is strengthened by the stimulatory effect of sodium NP, a spontaneous donor of NO, on LHRH release from MBH or arcuate/median eminence fragments *in vitro* (32, 42). In addition, NP that releases NO spontaneously has been shown to elevate cGMP levels in immortalized GnRH neurons and the hypothalamus (42). The mechanism of action of NO on GnRH stimulation appears to involve activation of the heme-containing enzyme, GC, the physiological target of exogenous and endogenous NO (42). Two types of GC, a membrane-bound or particulate and soluble form, have been identified (52, 53). Recently physiological concentrations of AA have been shown to inhibit both NO-dependent and NO-independent soluble GC without modifying the basal enzyme activity (48). The ability of AA to diminish accumulation of NO in the superfusate of isolated coronary arteries has been demonstrated, suggesting that AA also reduces NO, thereby inactivating it (54). Thus, not only does AA scavenge NO, but also it inhibits the activation of soluble GC by NO.

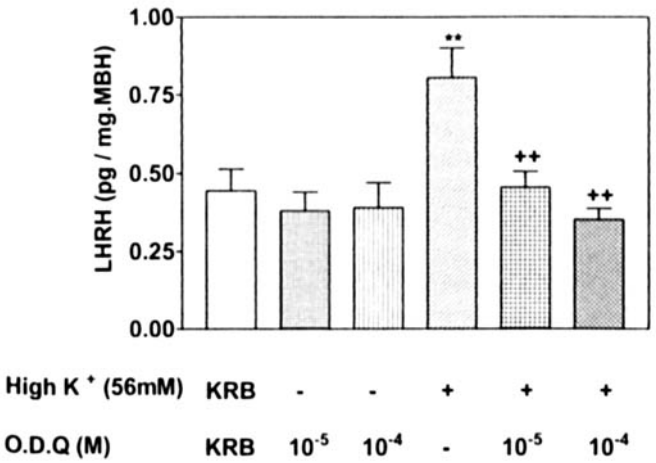

Figure 4. Influence of high [K^+] or combination of high [K^+] + O.D.Q. on LHRH release. **, $P < 0.01$ versus KRB. ++, $P < 0.01$ versus the group treated with high [K^+].

In the present investigation, we used cell membrane depolarization by high [K^+] and stimulation with NMDA to study the effect of LY 83583 and O.D.Q. on LHRH and AA release. Our previous experiments reported that high [K^+] stimulated AA, and LHRH release from MBH is abolished in the absence of calcium [Ca^{2+}], indicating that the release process is a [Ca^{2+}]-dependent phenomenon (37). High [K^+]-induced LHRH and AA release was inhibited by NMMA, a competitive inhibitor of NOS, showing that the release process involves NO. Taken together these results indicate that high [K^+]-induced AA and LHRH release is a [Ca^{2+}]-dependent and NO-mediated process. Membrane depolarization is known to activate [Ca^{2+}] influx through [Ca^{2+}] channels and activate NOS leading to the production of NO. NO would stimulate GC and activate protein kinase G that is required for the exocytosis of AA and LHRH.

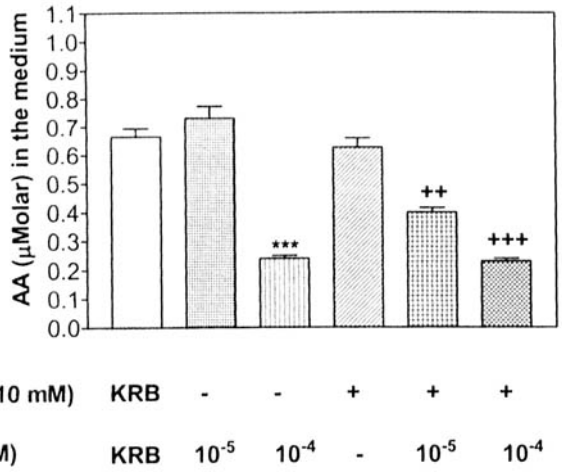

Figure 6. Effect of NMDA or combination of NMDA + O.D.Q. on AA release into the medium after I hr of incubation. ***, $P < 0.001$ versus KRB. ++, $P < 0.01$ or +++, $P < 0.001$ versus the group treated with NMDA.

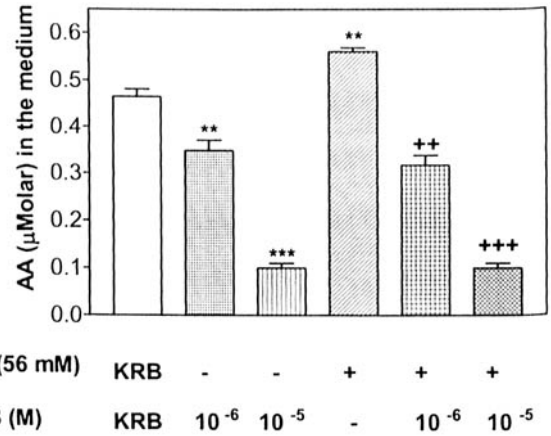

Figure 7. Effect of high [K^+] or combination of high [K^+] + LY 83583 on AA release into the medium. **, $P < 0.01$ or ***, $P < 0.001$ versus KRB. ++, $P < 0.01$ or +++, $P < 0.001$ versus the group incubated with high [K^+].

In the CNS, NOS is activated and synthesized in response to activation of the NMDA L-type glutamate receptors (18, 21, 22). Our previous experiments indicate that NMDA stimulates noradrenergic terminals that synapse on NOergic neurons and activate α_1–adrenergic receptors (32, 41). This activation causes an increase in intracellular free [Ca^{2+}] in the NOergic neurons and [Ca^{2+}] combines with calmodulin to activate NOS within these neurons. NO is synthesized by NOS from L-arginine and oxygen and in the presence of equimolar quantities of various cofactors and diffuses to the LHRH neuronal terminal, in which it activates GC that converts GTP to cGMP. Cyclic GMP is hypothesized to increase intracellular free [Ca^{2+}] that would participate in the exocytosis of LHRH secretory granules (32). Previously, NMDA has been shown to stimulate LHRH release from the hypothalamus, but at the dose used in this study (10 m*M*), there is no effect on AA release (37). However, a five times higher dose used in our earlier study has shown a significant decrease in AA release from MBH, suggesting that the larger quantities of NO formed by the higher dose oxidized AA (37).

Of the two inhibitors used in our study, LY 83583 suppressed neuronal NOS activity and reduced levels of NO-dependent cGMP in tissues of rats, indicating that its action is to inhibit NOS directly (44, 45, 55). On the other hand, O.D.Q. has been shown to act as a selective reversible, competitive inhibitor of the soluble GC targeted by NO both *in vivo* and *in vitro* (46, 47). However, it is not effective in suppressing particulate GC or adenylyl cyclase illustrating its specific action (46). In studies involving stimulation of brain slices with glutamate receptor agonist, NMDA, O.D.Q. neither interfered with any of the steps leading to NO synthesis nor inhibited NO release, supporting the view that its main target of action is soluble GC (46). In our experiment both concentrations of LY 83583 or O.D.Q. significantly lower high [K^+]-induced LHRH release without modifying the basal release. This suggests that cGMP may not play a role in basal LHRH release but exerts a stimulatory effect on high [K^+]-induced LHRH release.

Previously we showed that AA suppressed NMDA and NP-induced LHRH release (37). Because NMDA and NP have been shown to stimulate LHRH release via NO (32, 42, 43), we hypothesized that NO released from NOergic neurons oxidized AA to dehydro-AA, which cannot be measured by our method. Dehydro-AA is rapidly destroyed or reenters the tissue and is reduced to AA, and AA may chemically reduce NO as it is oxidized to dehydro-AA. The results for high [K^+] or NMDA-induced LHRH release are summarized in Figure 9. According to this hypothesis, the axons of the glutamergic neurons synapse on the axons of the noradrenergic neurons that in turn synapse on the NOergic neurons. Therefore, NMDA would cause the release of norepinephrine (NE) by α_1-noradrenergic receptors that would increase intracellular [Ca^{2+}] in the NOergic neuron. This increase would activate NOS, causing generation of NO

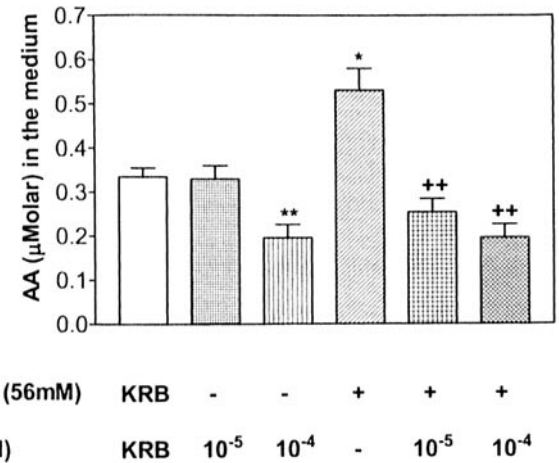

Figure 8. Effect of high [K^+] or combination of high [K^+] + O.D.Q. on AA release in the medium. *, $P < 0.05$ or **, $P < 0.01$ versus KRB. ++, $P < 0.01$ versus the group treated with high [K^+].

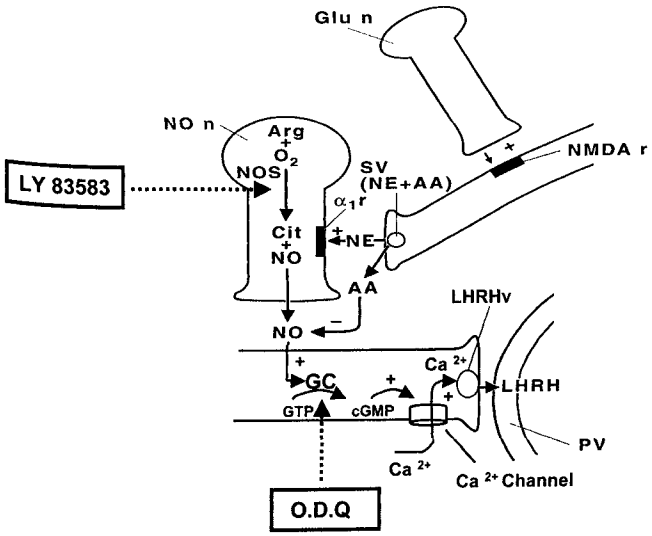

Figure 9. A schematic diagram of the hypothesized mechanism of action of LY 83583 and O.D.Q. on high [K^+] and NMDA-induced AA and LHRH release. Glu n, glutamergic neuron; NMDA r, NMDA receptor; SV, synaptic vesicle; α1 r, α1-adrenergic receptor; NO n, NOergic neuron; Arg, arginine; Cit, citrulline; gc, guanylyl cyclase; Ca^{2+}, calcium; +, stimulates; –, inactivates; LHRHv, LHRH vesicle; pv, portal vesicle; broken arrow, inhibition; LY 83583 and O.D.Q., NOS and GC inhibitor, respectively.

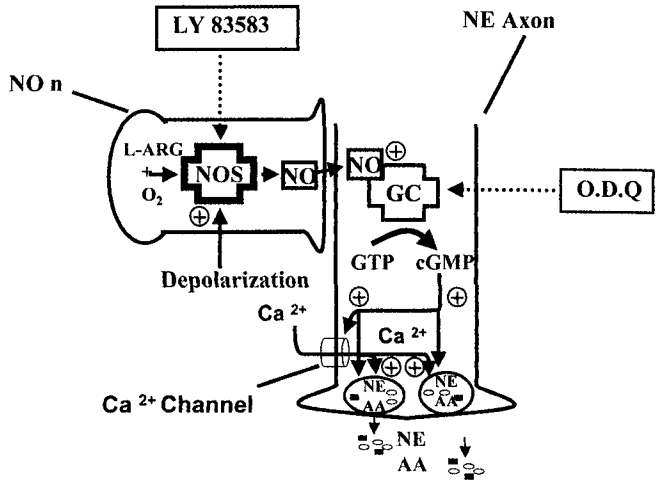

Figure 10. A schematic diagram of the hypothesized mechanism of action of LY 83583 and O.D.Q. on depolarization induced AA release. NO n, NOergic neuron; NE axon, norepinephrine axon; other abbreviations are described in Figure 9.

that would diffuse to the LHRH neuron and activate release by stimulating GC that converts GTP to cGMP.

Cyclic GMP is hypothesized to increase intracellular [Ca^{2+}] that would participate in the exocytosis of LHRH secretory granules. LY 83583 inhibited NOS, the enzyme required for the conversion of L-arginine to citrulline and NO. This is the initial step of the NO/cGMP signaling pathway. O.D.Q. inhibited soluble GC, the enzyme essential for the last reactions of the NO/cGMP signaling pathway, both drugs causing a decrease in cGMP. Studies with NMDA showed that it significantly stimulated LHRH release, and a total blockade of the stimulation was observed in the combined presence of either of the inhibitors.

However, the effect of LY 83583 and O.D.Q. on basal AA release is different as compared with that of LHRH. Both the inhibitors are effective in lowering basal AA release, although a 10 times higher concentration of O.D.Q. (10^{-4} *M*) is required to produce inhibition. These results support the hypothesis that cGMP is involved in basal AA release. The suppression of AA release is much greater in the combined presence of high [K^+] and LY 83583, suggesting that high [K^+] may evoke a greater release of intracellular [Ca^{2+}] that in turn activates NOS, thus causing a cascade of reactions ultimately resulting in increased cGMP. Suppression of basal release of AA as well as the inhibition of increased NOS activity in the presence of high [K^+] by LY 83583 may serve as the contributing factors for this decrease.

The results for depolarization induced AA release are hypothesized in Figure 10. We do not know the mechanism by which NO evokes depolarization-induced release of AA. However, membrane depolarization is known to activate [Ca^{2+}] influx through [Ca^{2+}] channels that may activate NOS leading to production of NO in NOergic neurons. NO in turn activates GC and would further increase intracellular free [Ca^{2+}] required for the exocytosis of AA and NE from the NE axon. Collectively, these results demonstrate that high [K^+]-induced AA release involve cGMP release. NMDA alone failed to alter AA release but in the presence of LY 83583 or O.D.Q., a significant decrease in AA release was observed, illustrating that NMDA-induced cGMP release was blocked by LY 83583 by its inhibition of NOS and O.D.Q. through its inhibition of GC.

LY 83583 has been shown to suppress neuronal NOS, and this may result in lower NO release that in turn lowers cGMP levels (56). O.D.Q., a selective inhibitor of the soluble GC, and LY 83583, an inhibitor of NOS, produced similar results. This suggests that the final effect with both the inhibitors is suppression of cGMP formation, although their targets are at different stages of the NO/cGMP signaling pathway. Our results further suggest that high [K^+]-induced AA and LHRH release involve NO activation of soluble GC and not particulate GC because O.D.Q. acts only on this form of the enzyme. These results have been obtained in static incubations and must be confirmed in a dynamic perfusion system to further test their validity. It would be very important to see whether the results will change in a situation in which AA has been depleted such as in scorbutic guinea pigs.

We would like to thank Judy Scott and Nicole Mestayer for their excellent secretarial assistance.

1. Rebec GV, Pierce RC. A vitamin as neuromodulator: ascorbate release into the extracellular fluid of the brain regulates dopaminergic and glutamatergic transmission (Review). Pro Neurobiol 43:537–565, 1994.

2. Spector R, Lorenzo AV. Ascorbic acid homeostasis in the central nervous system. Am J Phyiol 226:1468–1473, 1974.
3. Grunewald RA. Ascorbic acid in the brain. Brain Res Rev 18:123–133, 1993.
4. Spector R, Lorenzo AV. Ascorbic acid homeostasis in the central nervous system. Am J Physiol 225:757–763, 1973.
5. Chatterjee IB, Majumdar AK, Nandi BK, Subramanian N. Synthesis and some major functions of vitamin C in animals. Ann N Y Acad Sci 258:24–47, 1975.
6. Schenko IO, Miller E, Gaddis R, Adams RN. Homeostatic control of ascorbate concentration in CNS extracellular fluid. Brain Res 253:353–356, 1982.
7. Oke AF, May L, Adams RN. Ascorbic acid distribution patterns in human brain: comparison with nonhuman mammalian species (review). Ann N Y Acad Sci 498:1–12, 1987.
8. Agus DB, Gambhir SS, Pardridge WM, Spielholtz C, Baselga J, Vera JC, Gold DW. Vitamin C crosses the blood-brain barrier in the oxidized form through the glucose transporters. J Clin Invest 100: 2842–2848, 1997.
9. Washko P, Rotrosen D, Levine M. Ascorbic acid transport and accumulation in human neutrophils. J Biol Chem 264:18996–19002, 1989.
10. Tsukaguchi H, Tokui T, Mackenzie TB, Berger UV, Chen XZ, Wang Y, Brubaker RF, Hediger MA. A family of mammalian Na+-dependent L-ascorbic acid transporters. Nature 399:70–75, 1999.
11. Wang Y, Mackenzie B, Tsukaguchi H, Weremowicz S, Morton CC, Hediger MA. Human vitamin C (L-ascorbic acid) transporter SVCT1. Biochem Biophys Res Commun 267:488–494, 2000.
12. Chatterjee IB. Ascorbic acid metabolism (review). World Rev Nutr Diet 30:69–87, 1978.
13. Schreibe M, Trojan S. Ascorbic acid in the brain (review). Physiol Res 40:413–418, 1991.
14. Das PC, Das KP, Bagchi K, Dey CD. Evaluation of tissue ascorbic acid status in different hormonal states of female rat. Life Sci 52:1493–1498, 1993.
15. Palmer RM, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium derived relaxing factor. Nature 327:524–526, 1987.
16. Dawson TM, Snyder SH. Gases as biological messengers: nitric oxide and carbon monoxide in the brain. J Neurosci 14:5147–5159, 1994.
17. Schuman EM, Madison DV. Nitric oxide and synaptic function. Ann Rev Neuro 17:153–183, 1994.
18. Garthwaite J, Charles SJ, Chess-Williams R. Endothelium-derived relaxing factor release on activation of NMDA receptor suggests role as intercellular messenger in the brain. Nature 336:385–388, 1988.
19. Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology and pharmacology. Pharmacol Rev 43:109–142, 1991.
20. Garthwaite J. Glutamate, nitric oxide and cell-cell signaling in the nervous system. Trends Neurosci 14:60–67, 1991.
21. Knowles RG, Palacios M, Palmer RM, Moncada S. Formation of nitric oxide from L-arginine in the central nervous system: a transduction mechanism for stimulation of the soluble guanylate cyclase. Proc Natl Acad Sci U S A 86:5159–5162, 1989.
22. Bredt DS, Snyder SH. Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. Proc Natl Acad Sci U S A 86:9030–9033, 1989.
23. Moncada S, Higgs A. The L-arginine-nitric oxide pathway. N Engl J Med 329:2002–2012, 1993.
24. Ignarro LJ. Heme-dependent activation of guanylate cyclase by nitric oxide: a novel-signal transduction mechanism (review). Blood Vessels 28:67–73, 1991.
25. Johnstone MT, Creager SJ, Sceals KM, Cusco JA, Lee BK, Creager MA. Impaired-endothelium dependent vasodilation in patients with insulin-dependent diabetes mellitus. Circulation 88:2510–2516, 1993.
26. Chirkov YY, Chirkova LP, Horowitz JD. Suppressed anti-aggregating and cGMP-elevating effects of sodium nitroprusside in platelets from patients with stable angina pectoris. Naunyn Schmiedebergs Arch Pharmacol 354:520–525, 1996.
27. Kojda G, Kottenberg K, Hacker A, Noack E. Alterations of the vascular and the myocardial guanylate cyclase/cGMP-system induced by long term hypertension in rats. Pharm Acta Helv 73:27–35, 1998.
28. Creagar MA, Cooke JP, Mendelsohn ME, Gallagher SJ, Coleman SM, Loscalzo J, Dzau VJ. Impaired vasodilation of forearm resistance vessels in hypercholesterolemic humans. J Clin Invest 86:228–234, 1990.
29. Drexler H, Hayoz D, Munzel T, Just H, Zelis R, Brunner HR. Endothelial function in congestive heart failure (review). Am Heart J 126:761–764, 1993.
30. Taddei S, Virdis A, Ghiadoni L, Magagna A, Salvetti A. Vitamin C improves endothelium-dependent vasodilation by restoring nitric oxide activity in essential hypertension. Circulation 97:2222–2229, 1998.
31. Bhat G, Mahesh VR, Aguon K, Brann DW. Evidence that brain nitric oxide synthase is the major nitric oxide synthase isoform in the hypothalamus of the adult female rat and that nitric oxide potently regulates hypothalamic cGMP levels. Neuroendocrinology 64:93–102, 1996.
32. Rettori V, Belova N, Dees WL, Nyberg CL, Gimeno M, McCann SM. Role of nitric oxide in the control of luteinizing hormone releasing hormone release *in vitro*. Proc Natl Acad Sci U S A 90:10130–10134, 1993.
33. Mayer B, Koesling D, Bohne E. Characterization of nitric oxide synthase, soluble guanylyl cyclase and Ca2+/calmodulin-stimulated cGMP phosphodiesterase as components of neuronal signal transduction. Adv Second Messenger Phosphoprotein Res 28:111–119, 1993.
34. Valleboona F, Raiteri M. Monitoring of cyclic GMP during cerebellar microdialysis in freely-moving rats as an index of nitric oxide synthase activity. Neuroscience 57:577–585, 1993.
35. Uno H, Arakawa T, Fukuda T, Yu H, Fujiwara Y, Higuchi K, Inoue M, Kobayashi K. Nitric oxide stimulates prostaglandin synthesis in cultured rabbit gastric cells. Prostaglandins 53:153–162, 1997.
36. Rettori V, Gimeno M, Lyson K, McCann SM. Nitric oxide mediates norepinephrine-induced prostaglandin E_2 release from the hypothalamus. Proc Natl Acad Sci U S A 89:11543–11546, 1992.
37. Karanth S, Yu WH, Walczewska A, Mastronardi C, McCann SM. Ascorbic acid acts as an inhibitory transmitter in the hypothalamus to inhibit stimulated luteinizing hormone-releasing hormone release by scavenging nitric oxide. Proc Natl Acad Sci U S A 97:1891–1896, 2000.
38. Bonovera JJ, Sahu A, Kalra PS, Kalra SP. Evidence that nitric oxide may mediate the ovarian steroid induced luteinizing hormone surge: involvement of excitatory amino acids *in vitro*. Endocrinology 133:2481–2487, 1993.
39. Donoso AO, Lopez FJ, Negro-Vilar A. Glutamate receptors of the non-N-methyl D-aspartic acid type mediate the increase in luteinizing hormone releasing hormone release by excitatory amino acids *in vitro*. Endocrinology 126:414–420, 1990.
40. Bourguignon JP, Gerard A, Methieu J, Simons J, Franchimont P. Pulsatile release of gonadotropin releasing hormone from hypothalamic explants is restrained by blockade of N-methyl-d-aspartate receptors. Endocrinology 125:1090–1096, 1989.
41. Rettori V, Kamat A, McCann SM. Nitric oxide mediates the stimulation of luteinizing hormone releasing hormone release induced by glutamic acid in *vitro*. Brain Res Bull 33:501–503, 1994.

42. Moretto M, Lopez FJ, Negro-Vilar A. Nitric oxide regulates luteinizing hormone-releasing hormone secretion. Endocrinology 133:2399–2402, 1993.
43. Mahachoklertwattana P, Black SM, Kaplan SL, Bristow JD, Grumbach MM. Nitric oxide synthesized by gonadotropin releasing hormone neurons is a mediator of N-methyl-D-aspartate (NMDA) induced GnRH secretion. Endocrinology 135:1709–1712, 1994.
44. Diamond J, Chu EB. A novel cyclic GMP-lowering agent, LY 83583, blocks carbachol-induced cyclic GMP elevation in rabbit atrial strips without blocking the negative ionotropic effects of carbachol. Can J Physiol Pharmacol 68:908–911, 1985.
45. Schmidt MJ, Sawyer BD, Truex LL, Marshall WS, Fleisch JH. LY 83583: an agent that lowers intracellular levels of cyclic guanosine 3′5′-monophosphate. J Pharmacol Exp Ther 232:764–769, 1985.
46. Garthwaite J, Southam E, Boulton CL, Nielsen EB, Schmidt KB, Mayer B. Potent and selective inhibition of nitric oxide sensitive guanylyl cyclase by 1H-[1,2,4] oxadiazolo-[4,3-a] quinoxalin-1-one. Mol Pharmacol 48:184–188, 1995.
47. Fedele E, Jin Y, Varnier G, Raiteri M. *In vivo* microdialysis study of a specific inhibitor of soluble guanylyl cyclase on the glutamate receptor/nitric oxide/cyclic GMP pathway. Br J Pharmacol 119:590–594, 1996.
48. Schrammel A, Koesling D, Schmidt K, Mayer B. Inhibition of purified soluble guanylyl cyclase by L-ascorbic acid. Cardiovasc Res 47:602–608, 2000.
49. Harapanhalli RS, Howell RW, Rao DV. Testicular and plasma ascorbic acid levels in mice following dietary intake: a high performance liquid chromatographic analysis. J Chromatogr 614:233–243, 1993.
50. Petralia RS, Yokotani N, Wenthold RJ. Light and electron microscope distribution of the NMDA receptor subunit NMDA-R 1 in the rat nervous system using a selective antipeptide antibody. J Neurosci 14:667–696, 1994.
51. Bhat G, Mahesh VB, Lamar C, Ping L, Aguan K, Brann DW. Histochemical localization of nitric oxide neurons in the hypothalamus: association with gonadotropin-releasing hormone neurons and co-localization with N-methyl-D-aspartate receptors. Neuroendocrinology 62:187–197, 1995.
52. Chinkers M, Garbers DL. Signal transduction by guanylyl cyclases. Ann Rev Biochem 60:553–575, 1991.
53. Koestling D, Bohme E, Schultz G. Guanylyl cyclases, a growing family of signal transducing enzymes. FASEB J 5:2785–2791, 1991.
54. Murphy ME. Ascorbate and dehydroascorbate modulate nitric oxide-induced vasodilations of rat coronary arteries. J Cardiovasc Pharmacol 34:295–303, 1999.
55. Kamagai Y, Midorikawa K, Nakai Y, Yoshikawa T, Kushida K, Homma-Takeda S, Shimojo N. Inhibition of nitric oxide formation and superoxide generation during reduction of LY 83583 by neuronal nitric oxide synthase. Eur J Pharmacol 360:213–218, 1998.