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L-Citrulline recycling by argininosuccinate synthetase and lyase in rat gastric fundus

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Abstract

The aim of this study was to investigate in rat gastric fundus whether L-citrulline, the co-product in the nitric oxide (NO) biosynthesis catalyzed by neuronal nitric oxide synthase (nNOS), can be converted back to the nNOS substrate L-arginine. Immunohistochemistry showed that argininosuccinate synthetase and argininosuccinate lyase, that mediate transformation of L-citrulline to L-arginine in the ureum cycle in hepatocytes, co-localize with nNOS. In longitudinal smooth muscle strips, L-arginine as well as L-citrulline (10^{-3} M) was capable of completely respectively partially preventing the N^{G}-nitro-L-arginine methyl ester (L-NAME) (3×10^{-5} M)-induced inhibition of electrically induced nitrergic relaxations, whereas D-citrulline (10^{-3} M) was not. The L-citrulline-mediated prevention of the L-NAME-induced inhibition was reduced by L-glutamine (3×10^{-3} M), the putative L-citrulline uptake inhibitor, and by succinate, an argininosuccinate lyase inhibitor. The results demonstrate that the L-citrulline recycling mechanism is active in rat gastric fundus. Recycling of L-citrulline might play a role in providing sufficient amounts of nNOS substrate during long-lasting relaxations in gastric fundus after food intake.

Keywords: L-Citrulline recycling; Gastric fundus, rat; Argininosuccinate synthetase; Argininosuccinate lyase; Nitric oxide (NO) synthase, neuronal

1. Introduction

The role of nitric oxide synthase (NOS) in the production of the neurotransmitter nitric oxide (NO) is now well established. Three isoforms of NOS are described: an inducible NOS (iNOS), whose expression is induced after cytokine challenge, and two constitutive NOS isoforms, neuronal NOS (nNOS) and endothelial NOS (eNOS) (Stuehr, 1997). nNOS is held responsible for the generation of the non-adrenergic noncholinergic (NANC) inhibitory transmitter towards gastrointestinal smooth muscle (Lefebvre, 1995).

In the ureum cycle in hepatocytes, L-citrulline is transformed to L-arginine by the successive action of argininosuccinate synthetase and argininosuccinate lyase. The L-arginine is subsequently used by arginase to form L-ornithine and the cycle is completed with the transformation of L-ornithine to L-citrulline by ornithine transcarbamoylase (Morris, 2000). In cells producing NO, NOS catalyzes the formation of NO and L-citrulline. Some arguments suggest that L-citrulline is recycled to L-arginine in these NO-synthetising cells. Indeed, although the expression of the complete ureum cycle is only described in liver, argininosuccinate synthetase and argininosuccinate lyase are expressed in a whole range of cell types (Wu and Morris, 1998) and the induction of iNOS, that will lead to production of NO and thus L-citrulline, is accompanied by the enhancement of argininosuccinate synthetase expression in all nonhepatic cell types examined to date (Wu and Morris, 2000). Daniel et al. (2000) described the presence of argininosuccinate synthetase and argininosuccinate lyase and their co-localization with nNOS throughout the canine gastrointestinal tract. In the guinea-pig trachea, human bronchus and murine proximal colon, the inhibition of electrically induced nitrergic responses by competitive NOS inhibitors is overcome by both L-arginine and L-citrulline, the effect of the latter being ascribed to the conversion to L-arginine (Ellis and Conanan, 1994; Shuttleworth et al., 1997). We have now investigated the presence of the L-citrulline recycling system by immunohistochemical and functional experiments in rat gastric fundus, in which nitrergic relaxation has been established (Li and Rand, 1990; Boeckxstaens

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et al., 1991). In gastric fundus tissue, nNOS has to contribute to long-lasting relaxation, necessary upon food intake.

Arginase is just as argininosuccinate synthetase and argininosuccinate lyase also not exclusively expressed in liver. Two different isoforms of arginase exist: type I expressed in hepatocytes and type II expressed in nonhepatic cell types (Wu and Morris, 1998). Both arginase and NOS consume L-arginine and could therefore compete for the available substrate. Evidence for such a competition exists in cells where the expression of iNOS is induced (Hey et al., 1997; Wu and Morris, 1998). Also, for the constitutive NOS isoforms, substrate competition with arginase was reported. In penile corpus cavernosum (Cox et al., 1999) and internal anal sphincter (Baggio et al., 1999), inhibition of arginase results in an enhancement of nitrergic relaxation mediated by nNOS. In guinea-pig trachea, arginase inhibition results in a modulation of cholinergic airway reactivity via enhancement of cNOS activity (Meurs et al., 2000). However, until now, competition between arginase and nNOS in nitrergic nerves of the stomach has not been investigated. In this study, we therefore attempted to study the influence of arginase addition and inhibition on nitrergic relaxation in the rat gastric fundus.

2. Materials and methods

2.1. Tissue preparation

Male Wistar rats (250–420 g) were obtained from Janvier (Le Genest St. Isle, France). Rats were killed by a blow on the head and bleeding after 12 h of fasting with free access to water. Four longitudinal smooth muscle strips (approximately 15 mm long × 2 mm wide) were prepared from the gastric fundus as described by Vane (1957) and mounted under a tension of 1 g in 8.5 or 10 ml organ baths containing Krebs solution at 37 °C. The composition in mM was: NaCl 118.5, KCl 4.8, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.9, $NaHCO_3$ 25.0 and glucose 10.1. The Krebs solution contained 10^{-6} M atropine and 4×10^{-6} M guanethidine to block cholinergic and noradrenergic responses, respectively, and was bubbled with 95% O_2/5% CO_2. Tension was recorded auxotonically, via a Grass force displacement transducer FT03 coupled in series with a 1 g cm^{-1} spring, on a Graphtec linearcorder F WR3701 or Flatbed recorder (Kipp and Zonen). Electrical field stimulation was applied via 2 platinum plate electrodes (22 × 7 mm, distance in between 6 mm) by a Grass S88 stimulator with a constant voltage unit. The tissues were equilibrated for 1 h 30 with rinsing every 15 min.

2.2. Protocols

The influence of L-arginine, L-citrulline and D-citrulline on the inhibition of electrically induced NANC relaxation by N^G-nitro-L-arginine methyl ester (L-NAME) was studied as follows. After equilibration, tissues were contracted by 3×10^{-7} M prostaglandin $F_{2\alpha}$. Once a stable plateau was reached, tissues were stimulated electrically six times with 10 s trains (40 V, 2 Hz, 0.5 ms) at 5-min intervals. Tissues were then rinsed repetitively. Thirty-five minutes before a second prostaglandin $F_{2\alpha}$ plateau, L-arginine, L-citrulline or D-citrulline (10^{-3} M) was administered, followed 5 min later by L-NAME (3×10^{-5} M). During the second prostaglandin $F_{2\alpha}$ plateau, electrical field stimulation was repeated. To study the influence of drugs (L-glutamine, L-glutamate, L-aspartate, argininosuccinate, α methyl-D,L-aspartate (αMDLA), succinate) on the ability of L-citrulline to reduce the inhibitory effect of L-NAME, they were added 25 min before L-citrulline. Drugs were used at a concentration, which did not influence electrically induced relaxation per se.

To study the influence of exogenously applied arginase I (E.C. 3.5.3.1) or of L-norvaline (10^{-2} M) or *S*-(2-Boronoethyl)-L-Cysteine (BEC, 10^{-3} M), both arginase inhibitors, on electrically induced nitrergic relaxations, strips were incubated for 1 h with the enzyme or inhibitor before the second prostaglandin $F_{2\alpha}$ plateau.

The capacity of L-arginine and L-citrulline to reverse the L-NAME-induced inhibition was studied as follows. After equilibration, tissues were contracted by 3×10^{-7} M prostaglandin $F_{2\alpha}$. Once a stable plateau was reached, tissues were stimulated electrically 13 times with 10 s trains (40 V, 2 Hz, 0.5 ms) at 5-min intervals. After three control stimulations (stimulation 3 was set as reference), L-NAME was administered. After stimulation 7, L-arginine or L-citrulline (10^{-3} M) was added.

2.3. L-Arginine determinations

L-arginine was separated from the test tube material in the control set-up or from the extracts obtained after homogenization of gastric fundus tissue by a chromatographic procedure according to Chakder and Rattan (1997). Briefly, one part of extract or test tube material was diluted with three parts of 0.1 M citrate buffer (pH 5.3) and applied onto Dowex AG 50W × 8 resin columns (sodium form, 200–400 mesh, column volume in between 7 cm^3), that were pre-equilibrated with the same buffer. The columns were washed with 15 ml of citrate buffer and then eluted with 4 ml 0.2 N NaOH that quantitatively eluted the L-arginine from the columns.

L-Arginine levels of the elutes were determined by a colorimetric method using L-arginine as a standard (modified Sakaguchi reaction). The details have been described elsewhere (Chakder and Rattan, 1997). The whole procedure before measurement of the absorbance was performed on an ice bath. Briefly, 750 μl of sample was mixed with 125 μl of alkaline α-naphthol–thymine mixture (1:1 mixture of 0.04% α-naphthol in ethanol with 2% thymine in 10% NaOH). The samples were then treated with 50 μl of 4% NaOCl solution and mixed immediately. Exactly 1 min later, 50 μl of 2% $Na_2S_2O_3$ was added and mixed well. The

absorbance of the samples was measured at 515 nm using a spectrophotometer (Kontron Uvikon 930). Recovery of L-arginine from column purification was 75.1 ± 4.0% (mean ± S.E.M., $n = 16$) in the first series of L-arginine determinations and 101.0 ± 6.7% (mean ± S.E.M., $n = 9$) in the second series of L-arginine determinations. All L-arginine values are corrected for recovery.

The ability of arginase to consume L-arginine was assessed in a test tube control set-up under conditions identical to the functional set-up, i.e. in Krebs solution with 10^{-6} M atropine and 4×10^{-6} M guanethidine at 37 °C with continuous bubbling with 95% O_2/5% CO_2. In this solution, a concentration of 2.5×10^{-4} M L-arginine was incubated with aqua (control), arginase I 60 U /ml or L-norvaline 10^{-2} M plus arginase I 60 U/ml for 1 h. An additional series was performed with aqua (control), arginase I 60 U/ml or BEC (10^{-4}, 5×10^{-4} and 10^{-3} M) plus arginase I 60 U/ml for 1 h. Reaction was stopped by placing the reaction tubes on ice, immediately followed by L-arginine separation and measurement as described above.

Homogenates of longitudinal muscle strips of the rat gastric fundus were prepared according to Lefebvre et al. (1995) with minor modifications. Shortly, after incubation with aqua, L-NAME (3×10^{-5} M, 30 min), L-NAME (3×10^{-5} M, 30 min) plus L-citrulline (10^{-3} M, 35 min), arginase I (60 U/ml, 1 h), L-norvaline (10^{-2} M, 1 h) or BEC (10^{-3} M, 1 h), the tissue was quickly clamped between two liquid nitrogen cooled plates. After freezing, the tissue was homogenized with a mikro-dismembrator U (B. Braun, Melsungen, Germany) for 60 s. 1 ml of 6% trichloro acetic acid was then added and the material was then homogenized on ice with an ultrasonic probe (B. Braun Labsonic U) four times 7 s with a 15-s interval. After an incubation of 20 min on ice, the homogenate was centrifuged for 15 min at 3000 × g and 4 °C. The supernatant was extracted five times with 5 ml of water-saturated diethyl ether. L-Arginine in the

Table 1
Combination of primary, secondary and tertiary antibodies used for double labelling in cryocoupes of rat gastric fundus

Primary antibody combination			Visualization
Antibody	Supplier	Dilution	
Monoclonal mouse anti-PGP 9.5	Biogenesis	1:100	Combination 1
Polyclonal rabbit anti-nNOS	Sigma	1:5000	
Monoclonal mouse anti-argininosuccinate synthetase	Transduction Laboratories	1:100	Combination 1
Polyclonal rabbit anti-PGP 9.5	Biogenesis	1:6000	
Monoclonal mouse anti-argininosuccinate synthetase	Transduction Laboratories	1:100	Combination 1
Polyclonal rabbit anti-nNOS	Sigma	1:5000	
Monoclonal mouse anti-PGP 9.5	Biogenesis	1:100	Combination 2
Polyclonal rabbit anti-argininosuccinate lyase	gift M. Mori	1:500	
Monoclonal mouse anti-nNOS	Sigma	1:2000	Combination 2
Polyclonal rabbit anti-argininosuccinate lyase	gift M. Mori	1:500	

Combination	Secondary antibody			Tertiary antibody		
	Antibody	Supplier	Dilution	Antibody	Supplier	Dilution
1	Sheep anti-mouse biotinylated	Amersham	1:100	Streptavidin Cy3 labeled	Jackson Immunoresearch	1:10000
	Goat anti-rabbit FITC labeled	Jackson Immunoresearch	1:200			
2	Goat anti-mouse FITC labeled	Jackson Immunoresearch	1:100	Streptavidin Cy3 labeled	Jackson Immunoresearch	1:5000
	Goat anti-rabbit (F_{ab} fragment) biotinylated	Rockland	1:1000			

extract was separated and measured as described above. The pellet was resuspended in 0.2 N NaOH and protein content was determined according to the method of Bradford (1976) with the modification of Macart and Gerbaut (1982).

2.4. Immunohistochemistry

Rats were anaesthetized by intraperitoneal injection with nembutal (60 mg/kg) and transcardially perfused for 30 min with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, in mM: NaCl 225, NaH_2PO_4 20, Na_2HPO_4 80). Then, tissues were immediately isolated and fixed for 2 h at room temperature in 4% paraformaldehyde in 0.1 M PBS. Tissues were rinsed thoroughly in PBS, immersed overnight at 4 °C in 30% sucrose in PBS, embedded in Tissue-Tek OCT compound (Miles, Elkhart, IN, USA) and cut into 12-μm-thick cryostat sections which were mounted on chrome alum–gelatin-coated glass slides. Prior to immunolabeling, cryosections were pre-incubated with a blocking mixture containing 0.1% Triton-X-100, 0.5% thimerosal, 0.5% Bos-eral 20 T, 0.01% NaN_3 and 10% normal goat serum in 0.1 M PBS for 30 min at room temperature.

For double labelling, different combinations of primary antibodies were used as given in Table 1. Primary antibodies were incubated overnight in a humid chamber at room temperature. Visualization of the primary antisera was achieved by incubation with one of two secondary antibody combinations as required (see Table 1), followed by Cy 3-labeled streptavidin. Incubations for visualization were performed for 2 h in a humid chamber at room temperature.

Preparations were coverslipped with Citiphluor (Agar Scientific) and examined under a Zeiss Axiophot fluorescence microscope equipped with the appropriate filter set. Specificity of argininosuccinate synthetase and argininosuccinate lyase antibodies was confirmed in cryocoupes of rat kidney, where the antibodies specifically stained the lumen of the proximal convoluted tubules (Wu and Morris, 1998). Control experiments with the omission of the primary antibody did not result in staining in cryocoupes of rat gastric fundus and kidney.

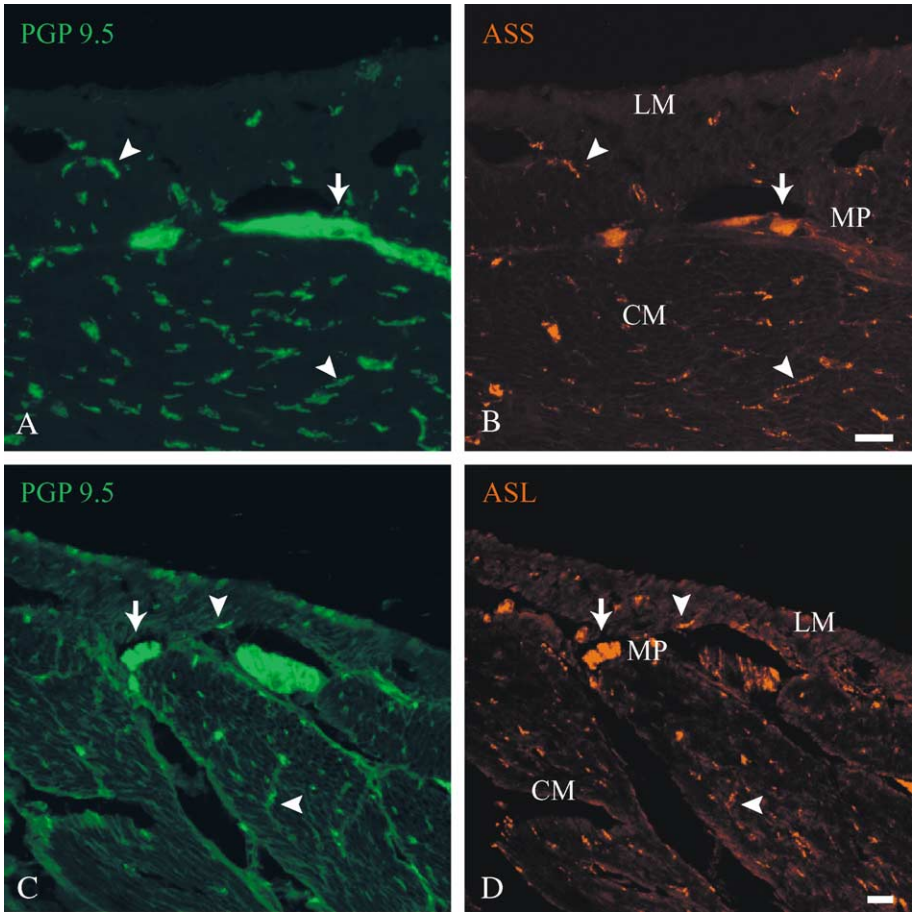

Fig. 1. Immunohistochemical staining for argininosuccinate synthetase, argininosuccinate lyase and PGP 9.5 in the longitudinal (LM) and circular (CM) smooth muscle layer and in the myenteric plexus (MP) of rat gastric fundus. Double labelling was performed for PGP 9.5 (A) and argininosuccinate synthetase (B) and for PGP 9.5 (C) and argininosuccinate lyase (D) and is represented on the same scale. The calibration bar stands for 20 μm. Double labelling for PGP 9.5 and argininosuccinate synthetase or argininosuccinate lyase was clearly visible in nerve cell bodies of the myenteric plexus (arrow) and in nerve fibers in muscle layers (arrowhead).

2.5. Drugs used

All chemicals, except for BEC, were obtained from Sigma (Bornem, Belgium). BEC was purchased from Qventas (Newark, USA). All drugs were dissolved in KREBS or deionised water.

2.6. Data analysis

Relaxations are expressed as percentage reduction of the prostaglandin $F_{2\alpha}$-induced tone. The percent decrease of the electrically induced relaxations by L-NAME in the different experimental conditions was calculated as $(R_{before} - R_{after}) \times 100/R_{before}$, where R_{before} and R_{after} indicate the response before and after the addition of L-NAME. Results are expressed as mean ± S.E.M. Results in two experimental conditions were compared by an unpaired *t*-test. Results in three or more experimental conditions were compared by one-way analysis of variance (ANOVA), followed by the Bonferroni procedure for multiple comparisons. Results within the same tissues were compared by a paired *t*-test. Statistical significance was accepted at $P < 0.05$. *n* Represents the number of results obtained in different animals.

3. Results

3.1. Presence and distribution of argininosuccinate synthetase and argininosuccinate lyase, protein-derived gene product (PGP) 9.5 and nNOS in rat gastric fundus

Immunoreactivity for PGP 9.5 was seen in the neurons of the myenteric plexus and in the nerve fibers running in the circular and longitudinal smooth muscle. Staining for nNOS resulted in a fluorescent signal in the same structures, be it that not all PGP 9.5 positive neuronal cell bodies and fibers stained for nNOS. All nNOS positive structures were, however, PGP 9.5 positive (results not shown).

Also argininosuccinate synthetase (Figs. 1B and 2B) and argininosuccinate lyase (Figs. 1D and 2D) immunos-

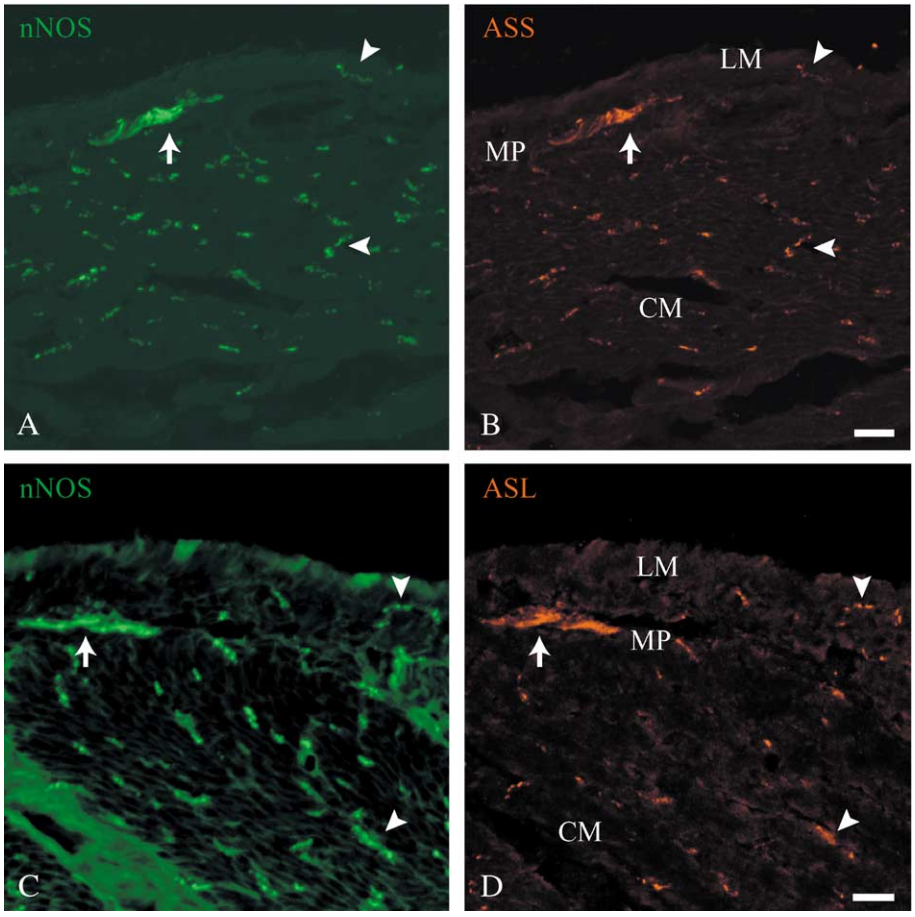

Fig. 2. Immunohistochemical staining for argininosuccinate synthetase, argininosuccinate lyase and nNOS in the longitudinal (LM) and circular (CM) smooth muscle layer and in the myenteric plexus (MP) of rat gastric fundus. Double labelling was performed for nNOS (A) and argininosuccinate synthetase (B) and for nNOS (C) and argininosuccinate lyase (D) and is represented on the same scale. The calibration bar stands for 20 μm. Double labelling for PGP 9.5 and argininosuccinate synthetase or argininosuccinate lyase was clearly visible in nerve cell bodies of the myenteric plexus (arrow) and in nerve fibers in muscle layers (arrowhead).

taining yielded a similar distribution pattern, i.e. in neurons of the myenteric plexus and in nerve fibers of both smooth muscle layers. The abundance of stained nerve fibers seen for all antigens tested in the circular smooth muscle layer was much less pronounced in the longitudinal smooth muscle layer.

Double labelling of argininosuccinate synthetase or argininosuccinate lyase with PGP 9.5 (Fig. 1A–B and C–D, respectively) showed that all argininosuccinate synthetase and lyase immunoreactivity was present in cells and fibers, which also stained for the neuronal marker PGP 9.5. However, not all PGP 9.5 positive neurons and nerve pro-

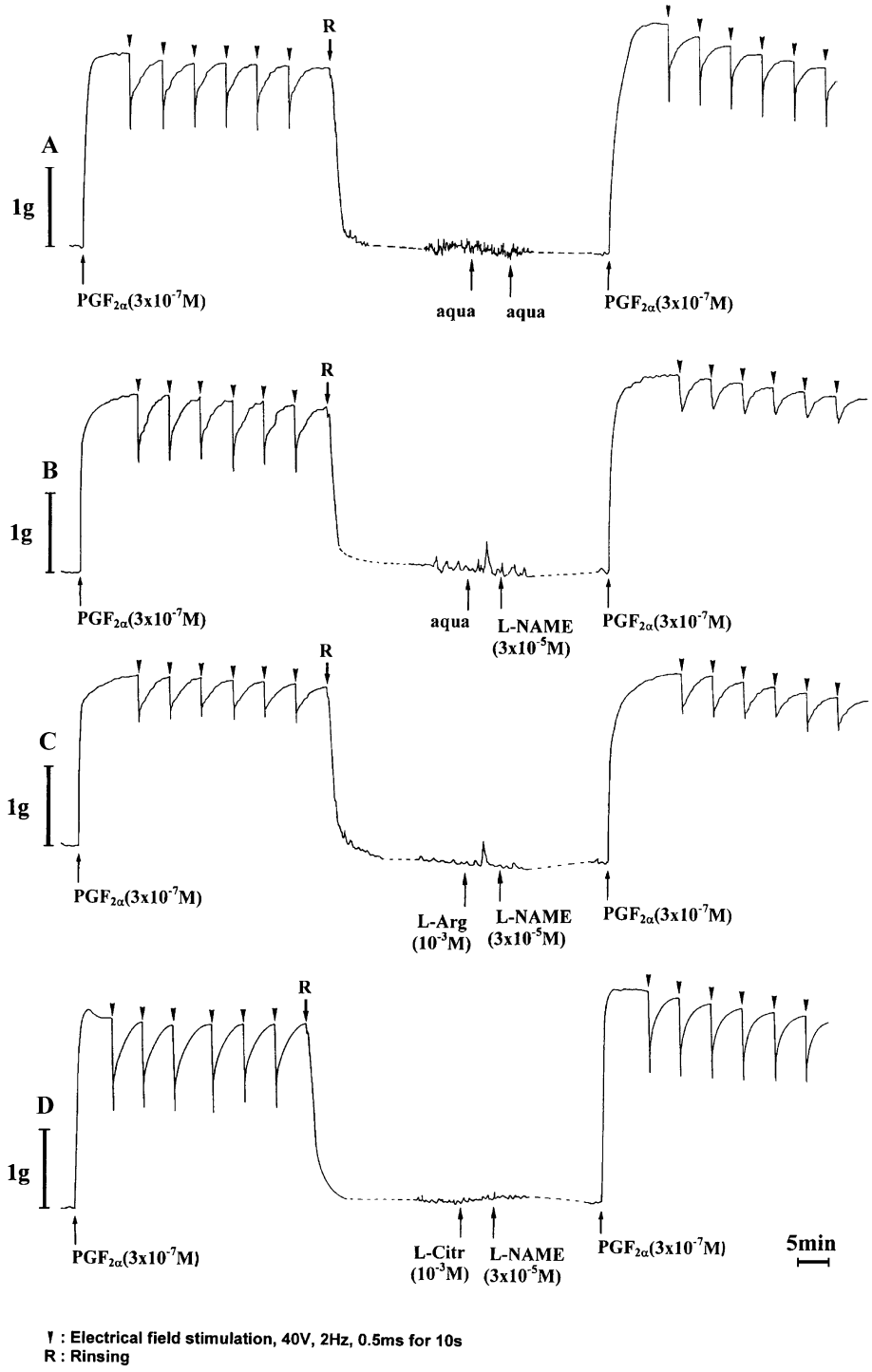

Fig. 3. Representative traces illustrating spontaneous inhibition (A) and the L-NAME (3×10^{-5} M)-induced inhibition (B) of the relaxation elicited by electrical field stimulation in longitudinal smooth muscle strips of the rat gastric fundus, contracted by administration of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). L-Arginine (L-Arg) (C) and L-citrulline (L-Citr) (D) partially prevented the inhibitory effect of L-NAME.

files were argininosuccinate synthetase or argininosuccinate lyase positive. Double labeling of argininosuccinate synthetase or argininosuccinate lyase with nNOS (Fig. 2A–B and C–D, respectively) showed a nearly complete overlap.

3.2. Prevention of the L-NAME-induced inhibition of electrically induced relaxation by L-arginine and L-citrulline

Electrical field stimulation induced a relaxation of the prostaglandin $F_{2\alpha}$-induced contraction level. The relaxant response was stable over the six stimulation trains within each plateau, but a moderate reduction of electrically induced relaxation was observed when examined on the second prostaglandin $F_{2\alpha}$-induced contraction plateau (Fig 3A), reaching significance at stimulations 1 ($20 \pm 5\%$ reduction, $P<0.05$), 4 ($22 \pm 5\%$ reduction, $P<0.05$) and 6 ($15 \pm 5\%$ reduction, $P<0.05$) ($n=7$). L-NAME (3×10^{-5} M) inhibited electrically induced relaxations by approximately 50% to 60% at the six stimulations (Fig. 3B). This inhibition was significantly more pronounced than the spontaneous decline in the control tissues. Pre-incubation with L-arginine (10^{-3} M) completely prevented the L-NAME-induced inhibition (Figs. 3C and 4A), and this prevention was stable over the six stimulation trains. For the first stimulation, the inhibition by L-NAME was reduced from $51 \pm 6\%$ to $21 \pm 6\%$ ($n=6$) ($P<0.01$); for the sixth stimulation, the inhibition by L-NAME was reduced from $61 \pm 5\%$ to $26 \pm 3\%$ ($n=7$) ($P<0.01$). The inhibition still observed in the presence of L-arginine plus L-NAME was not significantly different from the spontaneous inhibition. L-Citrulline (10^{-3} M) was also capable of significantly preventing the L-NAME-induced inhibition, although to a smaller extent than L-arginine (Figs. 3D and 4B). The L-citrulline-mediated prevention was significant in five out of six stimulation trains. For the first stimulation, the inhibition by L-NAME was reduced from $55 \pm 6\%$ to $31 \pm 5\%$ ($n=7$) ($P<0.05$); for the sixth stimulation, the inhibition by L-NAME was reduced from $60 \pm 6\%$ to $39 \pm 4\%$ ($n=7$) ($P<0.05$).

A higher concentration of L-NAME (10^{-4} M) induced a significantly higher inhibition of the electrically induced NANC relaxations, e.g. for the first stimulation, the inhibition mediated by L-NAME (3×10^{-5} M) was $52 \pm 4\%$ ($n=13$) and by L-NAME (10^{-4} M) was $61 \pm 5\%$ ($n=14$) ($P<0.05$). L-Arginine (10^{-3} M) was still able to reduce the inhibitory effect of L-NAME (10^{-4} M) significantly at stimulations 3 to 6, e.g. for the third stimulation, the inhibition by L-NAME was reduced from $63 \pm 4\%$ to $37 \pm 9\%$ ($n=6$) ($P<0.05$). The L-arginine-mediated prevention of the L-NAME (10^{-4} M)-induced inhibition was significantly less pronounced than the prevention of the L-NAME (3×10^{-5} M)-induced inhibition. L-Citrulline (10^{-3} M) significantly reduced the effect of L-NAME (10^{-4} M). The L-citrulline-mediated prevention of the L-NAME (10^{-4} M)-induced inhibition tended to be lower than that of the L-NAME (3×10^{-5} M)-induced inhibition, but this did not reach significance. Still, for this reason, the following experiments were performed with 3×10^{-5} M L-NAME.

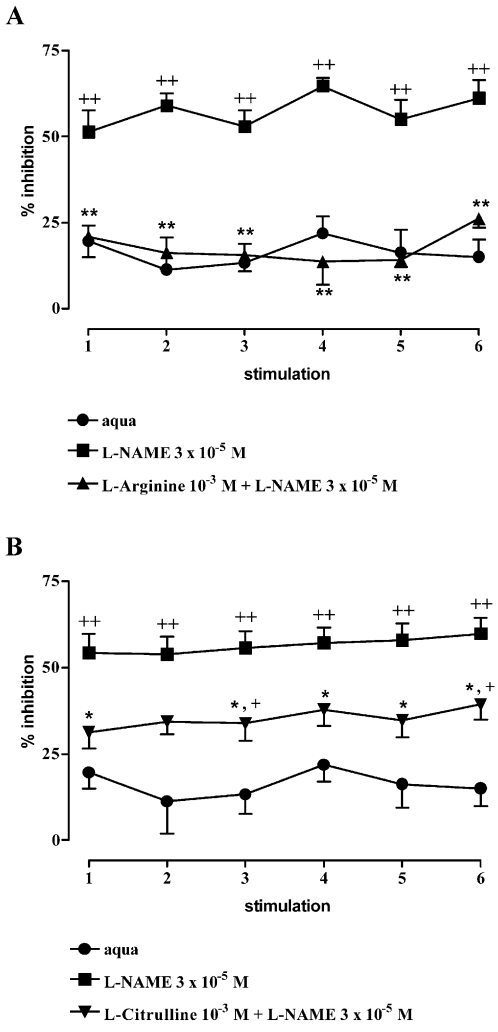

Fig. 4. Prevention of the L-NAME (3×10^{-5} M)-induced inhibition of nitrergic relaxation by 10^{-3} M L-arginine (A) and 10^{-3} M L-citrulline (B). Results are means ± S.E.M. of seven experiments. $^{+}P<0.05$, $^{++}P<0.01$ significantly different from the spontaneous inhibition in the absence of L-NAME. $^{*}P<0.05$, $^{**}P<0.01$ significantly different from the inhibition mediated by L-NAME alone.

The ability of L-arginine (10^{-3} M) and L-citrulline (10^{-3} M) to reverse the L-NAME (3×10^{-5} M)-induced inhibition was also tested. In control tissues, the relaxant response to the 13 electrical stimulations showed a progressive moderate decline (Fig. 5A). L-NAME inhibited the relaxant response to electrical stimulation (Fig. 5B–D). The inhibitory effect of L-NAME was maintained (Fig. 5B). L-Arginine partially reversed the inhibitory effect of L-NAME (Fig. 5C). Also L-citrulline tended to do so but was less effective than L-arginine (Fig. 5D).

L-Arginine (10^{-3} M) or L-citrulline (10^{-3} M) did not induce relaxation per se; they did also not enhance electrically induced NANC relaxations (results not shown).

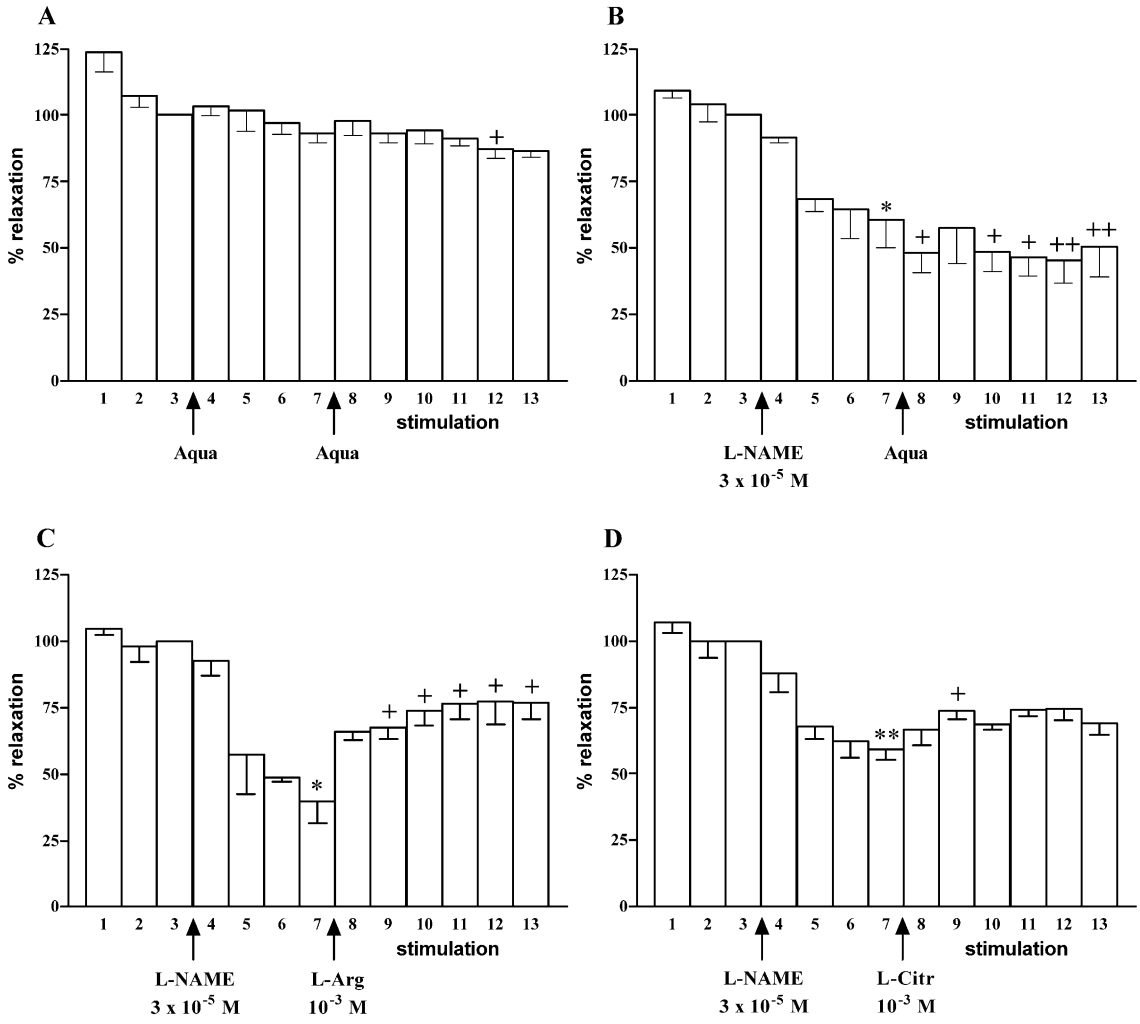

Fig. 5. Reversal of L-NAME (3×10^{-5} M)-induced inhibition of nitrergic relaxation by L-arginine (L-Arg) (C) and L-citrulline (L-Citr) (D). Longitudinal smooth muscle strips of the rat gastric fundus were electrically stimulated 13 times with 10-s trains (40 V, 2 Hz, 0.5 ms) at 5-min intervals. L-NAME was administered before the 4th stimulation and L-arginine or L-citrulline before the 8th stimulation. In control tissues (A, B), aqua was added at the adequate moments. Results are means ± S.E.M. of six experiments. Relaxation is expressed as percentage of the relaxation obtained at stimulation 3. $^{*}P<0.05$, $^{**}P<0.01$ significantly different from the relaxation at stimulation 3; $^{+}P<0.05$, $^{++}P<0.01$ significantly different from the relaxation at stimulation 7.

3.3. Influence of D-citrulline, L-glutamine, L-glutamate, L-aspartate and argininosuccinate on the L-citrulline-mediated prevention of the L-NAME-induced inhibition

To test the stereo-specificity of the L-citrulline-mediated prevention of the L-NAME-induced inhibition, the effect of D-citrulline was studied. D-Citrulline (10^{-3} M) did not change the inhibitory effect of L-NAME ($n=6$).

L-Glutamine (3×10^{-3} M), the putative L-citrulline uptake inhibitor, significantly reduced the prevention of the L-NAME-induced inhibition caused by L-citrulline ($P<0.01$ at stimulation 1) while L-glutamate (3×10^{-3} M) did not have such effect (Fig. 6). At stimulation 2, L-glutamate even significantly enhanced the L-citrulline-mediated prevention of the L-NAME-induced inhibition ($P<0.05$).

The first step in the L-citrulline recycling pathway is catalyzed by argininosuccinate synthetase and involves the condensation of L-citrulline and L-aspartate to form L-argininosuccinate. Pretreatment of tissues with a combination of L-aspartate (10^{-3} M) and L-citrulline (10^{-3} M) before L-NAME (3×10^{-5} M) did not enhance the protective effect of L-citrulline on the L-NAME-induced inhibition ($n=8$). Also, pre-incubation with argininosuccinate (10^{-3} M) did not influence the L-NAME-induced inhibition of the NANC relaxation ($n=6$).

3.4. Influence of the argininosuccinate synthetase inhibitor αMDLA and the argininosuccinate lyase inhibitor succinate on the L-citrulline-mediated prevention of the L-NAME-induced inhibition

αMDLA is reported as an inhibitor of argininosuccinate synthetase in vitro (Morris, 2000; Keilhoff et al., 2000). 3×10^{-3} M αMDLA however did not change the L-citrulline-mediated prevention of the L-NAME-induced inhibition

in rat gastric fundus longitudinal smooth muscle strips ($n=4$).

A study of Lee et al. (1992) reported the inhibition of argininosuccinate lyase by succinate, as an analogue of its substrate argininosuccinate. As shown in Fig. 7, the prevention mediated by L-citrulline on the L-NAME-induced inhibition is significantly reduced in the presence of succinate (3×10^{-3} M) ($P<0.05$) at stimulations 5 and 6.

3.5. Influence of arginase treatment on tissue L-arginine levels and on electrically induced relaxation

Arginase I was able to metabolize L-arginine, dissolved in Krebs solution with atropine and guanethidine, in the test tube control set-up. In the control tube with 2.5×10^{-4} M L-arginine, the percentage of L-arginine detected in the column eluate was $109.7\pm6.7\%$ of the amount added; in the presence of arginase I (60 U/ml), this was reduced to $0.0\pm1.0\%$ ($n=5$) ($P<0.01$). In the presence of arginase I (60 U/ml) plus L-norvaline (10^{-2} M), this percentage increased to $69.5\pm16.7\%$ ($n=5$, $P<0.01$). In a second series of experiments, BEC was tested as inhibitor of arginase I in a test tube control set-up. $117.7\pm6.8\%$ of the added L-arginine was detected in the column eluate ($n=5$). In the presence of arginase I (60 U/ml), this was reduced to $6.2\pm1.7\%$ ($n=5$) ($P<0.01$). BEC concentration-dependently increased the amount of L-arginine detected in the column eluate: at 10^{-4} M $13.4\pm4.5\%$, at 5×10^{-4} M $61.0\pm10.8\%$ ($P<0.01$) and at 10^{-3} M $95.6\pm16.2\%$ ($P<0.01$) ($n=5$).

L-Arginine content of longitudinal smooth muscle strips was not changed after arginase treatment: $(4.2\pm0.8)\times10^{-10}$ mol L-arginine/mg tissue in controls versus $(4.1\pm0.8)\times10^{-10}$ mol L-arginine/mg tissue in arginase-treated strips ($n=6$). Accordingly, also NANC relaxations were not reduced after incubation with 60 U/ml arginase I.

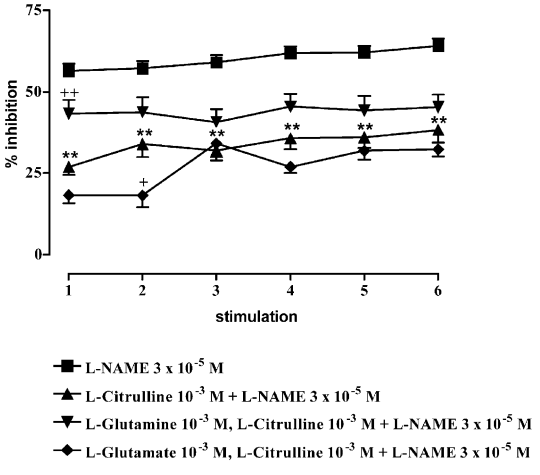

Fig. 6. Effect of L-glutamine (3×10^{-3} M) and L-glutamate (3×10^{-3} M) on the L-citrulline (10^{-3} M)-mediated prevention of the L-NAME (3×10^{-5} M)-induced inhibition. Results are means ± S.E.M. of eight experiments. $^{**}P<0.01$ significantly different from the inhibition mediated by L-NAME alone; $^{+}P<0.05$, $^{++}P<0.01$ significantly different from the inhibition mediated by L-NAME in the presence of L-citrulline.

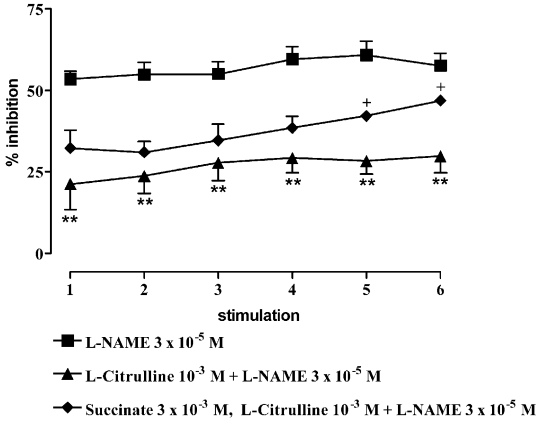

Fig. 7. Effect of succinate (3×10^{-3} M) on the L-citrulline (10^{-3} M) mediated prevention of the L-NAME (3×10^{-5} M)-induced inhibition. Results are means ± S.E.M. of eight experiments. $^{**}P<0.01$ significantly different from the inhibition mediated by L-NAME alone; $^{+}P<0.05$ significantly different from the inhibition mediated by L-NAME in the presence of L-citrulline.

The L-arginine content of longitudinal smooth muscle strips was also not changed after L-norvaline (10^{-2} M) or BEC (10^{-3} M) treatment: $(3.0\pm1.0)\times10^{-10}$ mol L-arginine/mg tissue in controls versus $(3.3\pm0.3)\times10^{-10}$ mol L-arginine/mg tissue in L-norvaline-treated strips ($n=4$) and $(4.6\pm0.6)\times10^{-10}$ mol L-arginine/mg tissue in controls versus $(4.5\pm0.4)\times10^{-10}$ mol L-arginine/mg tissue in BEC-treated strips ($n=4$). L-Norvaline (10^{-2} M) nor BEC (10^{-3} M) did influence electrically induced NANC relaxations ($n=6$).

Finally, L-arginine levels were determined in strips treated with L-NAME or L-citrulline plus L-NAME. Values were $(3.8\pm0.8)\times10^{-10}$ mol L-arginine/mg tissue in L-NAME-treated strips ($n=6$) and $(4.0\pm0.8)\times10^{-10}$ mol L-arginine/mg tissue in L-citrulline plus L-NAME-treated strips ($n=6$).

4. Discussion

The aim of this study was to investigate if the L-citrulline recycling mechanism was active in nitrergic neurons in the rat gastric fundus. The immunohistochemical results clearly demonstrate the co-localization of nNOS together with argininosuccinate synthetase and argininosuccinate lyase in the neurons of the myenteric plexus and in the nerve fibers of the circular and longitudinal smooth muscle. The fact that the distribution of argininosuccinate synthetase and argininosuccinate lyase closely mimics that of nNOS suggests that L-citrulline can be converted to L-arginine through the serial catalysis by argininosuccinate synthetase and argininosuccinate lyase in nitrergic neurons. Their action would maintain a supply of L-arginine to be used as a substrate for nNOS. Indeed, the tissue under study, gastric fundus, is well known for its

large and long-lasting relaxation upon food intake. The argininosuccinate synthetase and argininosuccinate lyase co-localization with nNOS in nitrergic neurons correlated with results from other tissues. Yu et al. (1997) described co-localization of argininosuccinate synthetase, argininosuccinate lyase and nNOS immunoreactivity in perivascular nerves, while also throughout the canine gastrointestinal tract, argininosuccinate synthetase and argininosuccinate lyase were co-localized with nNOS in the same neurons and neuronal fibers (Daniel et al., 2000). Also in bovine aortic endothelial cells, cellular colocalization of enzymes involved in NO production and arginine regeneration was demonstrated (Flam et al., 2001).

The NANC relaxation of rat gastric fundus in response to electrical stimulation at 2 Hz for 10 s was largely nitrergic as evident from the inhibitory effect of L-NAME, confirming previous results (Li and Rand, 1990; Boeckxstaens et al., 1991). The inhibition by L-NAME was completely overcome by the substrate L-arginine as expected for a competitive NOS inhibitor. Also L-citrulline clearly prevented the inhibition by L-NAME, although the effect was less pronounced than with L-arginine. The observation that D-citrulline did not mimic the effect of L-citrulline corresponds with the idea that L-citrulline is active through conversion to L-arginine by argininosuccinate synthetase and argininosuccinate lyase, that do not use the D-enantiomers as substrate. The preventing effect of L-citrulline versus L-NAME was maintained for six stimulations at 5-min interval. This contrasts with the results of Shuttleworth et al. (1997), who observed a transient L-citrulline effect when used at a concentration of 2×10^{-3} M in contrast with a sustained effect at 2×10^{-4} M L-citrulline.

The recycling mechanism of L-citrulline was pharmacologically further elaborated using drugs, aimed at interfering with L-citrulline uptake or metabolism. If exogenous L-citrulline mediates its prevention by recycling towards L-arginine via the proposed pathway with the cellular enzymes argininosuccinate synthetase and argininosuccinate lyase, L-citrulline needs to be taken up by the cell. L-Glutamine has been shown to inhibit neuronal L-citrulline uptake. In an electron-microscopic autoradiographic study (Yu et al., 1997), decrease in dense silver staining grains representing L-[^{3}H]citrulline uptake in the perivascular nerves was indeed obtained after L-glutamine pretreatment. In this study, L-glutamine moderately inhibited the L-citrulline-mediated prevention of the L-NAME-induced inhibition, while the similar amino acid, L-glutamate, did not have such an effect, correlating with the results of Chakder and Rattan (1997) in opossum internal anal sphincter. Although αMDLA was reported as an inhibitor of argininosuccinate synthetase when tested in vitro in a test tube (Ratner, 1973) and in rat brain slices (Keilhoff et al., 2000), it was not able to reduce the L-citrulline effect on the L-NAME-induced inhibition in our study. The argininosuccinate lyase inhibitor succinate (Lee et al., 1992), in contrast, did reduce the L-citrulline-mediated prevention of the L-NAME-induced inhibition, supporting recycling of L-citrulline to L-arginine.

We further tested the possible influence of L-aspartate and L-argininosuccinate. The first step in the L-citrulline recycling pathway is catalyzed by argininosuccinate synthetase and involves the condensation of L-citrulline and L-aspartate to form L-argininosuccinate. The effect of L-citrulline might therefore be limited by insufficient availability of L-aspartate. However, pretreatment with L-citrulline in combination with L-aspartate did not result in an enhancement of the protective effect of L-citrulline, suggesting that L-aspartate is present in sufficient amounts. Similarly, in murine proximal colon, no additional effect of L-aspartate on the L-citrulline-mediated prevention of the inhibition of the nitrergic response was seen (Shuttleworth et al., 1997). As an intermediate in the recycling pathway, L-argininosuccinate could theoretically substitute for L-citrulline in the recycling towards L-arginine. However, it was not able to prevent the L-NAME-induced inhibition. Possibly, an activation signal originating from the first step in the L-citrulline recycling is needed for argininosuccinate lyase to catalyze the second step in the recycling. The lack of L-argininosuccinate to prevent L-NAME-induced inhibition corresponds with the results obtained in internal anal sphincter (Rattan and Chakder, 1997), where L-arginine and L-citrulline but not L-argininosuccinate were capable of restoring electrically induced NANC relaxation after L-arginine depletion. These authors suggested the absence of a specific transporter for uptake of L-argininosuccinate in the myenteric neuronal cytosol.

The moderate effect of L-glutamine (inhibitor of L-citrulline uptake) and of succinate (inhibitor of argininosuccinate lyase) and the non-effect of αMDLA (inhibitor of argininosuccinate synthetase) might raise concern about the functional importance of L-citrulline recycling in rat gastric fundus. Still, there was a nearly complete colocalization of nNOS with the L-citrulline recycling enzymes and throughout all series, L-citrulline consistently reduced the L-NAME-induced inhibition. Very few pharmacological tools are available to influence the L-citrulline recycling pathway and the moderate to non-effect of the substances used might be related to weak specificity and efficacy. L-Glutamine has recently been used as a L-citrulline uptake inhibitor by different authors (Ellis and Conanan, 1994; Chakder and Rattan, 1997; Yu et al., 1997) but results varied from no effect on Ellis and Conanan (1994) to complete block of L-citrulline-mediated reversion of NOS inhibition (Chakder and Rattan, 1997). Ellis and Conanan (1994) suggested that the efficiency of L-glutamine is cell type dependent. Some authors suggest that the action of L-glutamine relies on inhibition of argininosuccinate synthetase rather than on inhibition of L-citrulline uptake (Su and Block, 1995; Morris, 2000; Raghavan and Dikshit, 2001).

Succinate was reported as an inhibitor of purified bovine argininosuccinate lyase in a study of Lee et al. (1992). However, no thorough kinetic study on the inhibitory action was performed, so data on specificity and efficacy of succinate are missing. Although αMDLA has been reported to inhibit argininosuccinate synthetase in the test tube (Ratner, 1973), its specificity in intact cells has been questioned (Morris, 2000). The only mammalian enzyme known to use L-citrulline to date is argininosuccinate synthetase. The product of the latter, argininosuccinate, can only be metabolized by argininosuccinate lyase. We thus believe that the effect of L-citrulline on the L-NAME-induced inhibition of nitrergic response in the rat gastric fundus is due to the metabolization of L-citrulline to L-arginine by the combined action of argininosuccinate synthetase and lyase. Still, the functional assessment of endogenous L-arginine synthesis via L-citrulline recycling remains difficult due to the lack of highly specific inhibitors, as already put forward by other authors (Morris, 2000).

Although L-arginine was also able to reverse L-NAME-induced inhibition of the electrically induced NANC relaxation, L-citrulline only had a very moderate effect in this protocol. We have no clear cut explanation for this observation. L-Citrulline as well as L-arginine had no relaxant effect per se, illustrating that L-arginine is not a limiting factor for the electrically induced NANC relaxations. We also observed that, although L-citrulline is capable of preventing the L-NAME-induced inhibition, no enhancement of bulk L-arginine concentration was detected. However, measurement of bulk L-arginine concentration can overlook smaller compartmental changes. Indeed, L-arginine used as a substrate for NOS might originate from a unique, separate pool, as suggested for eNOS (Flam et al., 2001; Kim et al., 2001; Hecker et al., 1990). Furthermore, also in the study of Flam et al. (2001), regeneration of L-arginine did not affect bulk intracellular concentrations. Our observations that L-citrulline and L-arginine had no relaxant effect per se correspond to the observations of Chakder and Rattan (1997) in opossum internal anal sphincter. Raghavan and Dikshit (2001) describe a relaxant effect of L-citrulline in rat aorta, suggested to be the consequence of extra supply of NOS substrate after the conversion of L-citrulline to L-arginine. Corresponding with this hypothesis, also L-arginine induced relaxation with an almost overlapping concentration-response curve with L-citrulline. Although rat aorta has been reported to contain similar L-arginine amounts as those measured in gastrointestinal and other tissues (see following paragraph), these amounts in vascular tissue apparently do not support full NOS activity and L-arginine therefore induces relaxation. The relaxation by L-citrulline in endothelium-denuded rabbit aorta rings (Ruiz and Tejerina, 1998), in contrast, was unaffected by L-NAME or the soluble guanylate cyclase inhibitor ODQ and was ascribed to a particulate guanylate cyclase-dependent pathway, suggesting that species differences exist within the same tissue.

We also aimed at investigating the possible competition between nNOS and arginase for the substrate L-arginine in the rat gastric fundus. The L-arginine levels in the rat gastric fundus are similar to those reported in different tissues such as opossum internal anal sphincter (Chakder and Rattan, 1997), mouse liver and brain (de Jonghe et al., 2001), rat liver, lungs, aorta and heart (Schott et al., 1993; Lortie et al., 2000) and in human benign breast and colon tumors (Park et al., 1991). Although the arginase used (arginase I) was able to consume L-arginine in a test tube, we were not able to induce L-arginine depletion in the rat gastric fundus. This contrasts with the results obtained in the opossum internal anal sphincter (Chakder and Rattan, 1997), although the same concentration of arginase and the same incubation period was used. Also L-norvaline, an arginase inhibitor (Meurs et al., 2000), significantly reduced arginase activity as measured in the test tube, but did not alter L-arginine content or the nitrergic responses of the strips. The same applied for BEC, which was even a more potent inhibitor of arginase in the test tube. Several explanations for this result can be put forward. Firstly, L-norvaline (10^{-2} M) and BEC (10^{-3} M) are effective in inhibiting arginase I as used in the test tube, but may be not for arginase II, the isoform presumably present in the rat gastric fundus. Secondly, rat gastric fundus may contain no or very limited amounts of arginase II, whereby inhibition has no or only minimal effect. Thirdly, L-norvaline is not able to reach endogenous arginase. As for BEC, diffusion problems are not expected since the enhancement of NANC responses of rabbit and human penile corpus cavernosum in a comparable organ bath set-up is reported (Kim et al., 2001). Arginase I, L-norvaline and BEC did not influence electrically induced NANC relaxations. This might still be the case if these agents would be able to change the L-arginine concentrations, as L-arginine per se did not affect electrically induced NANC relaxations, illustrating that endogenous L-arginine is not a limiting factor for the electrically induced NANC relaxation. Since pure arginase II or an antibody against arginase II is not commercially available, this line of investigation could not be further elaborated.

In conclusion, the L-citrulline recycling system is present and active in rat gastric fundus longitudinal muscle. L-Citrulline recycling in rat gastric fundus tissue might be important in order to maintain sufficient amounts of L-arginine during long-lasting relaxation after food intake.

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