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Regulation of citrulline recycling in nitric oxide-dependent neurotransmission in the murine proximal colon

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1 We investigated the contribution of nitric oxide (NO) to inhibitory neuromuscular transmission in murine proximal colon and the possibility that citrulline is recycled to arginine to maintain the supply of substrate for NO synthesis.

2 Intracellular microelectrode recordings were made from circular smooth muscle cells in the presence of nifedipine and atropine (both 1 μM). Electrical field stimulation (EFS, 0.3–20 Hz) produced inhibitory junction potentials (i.j.ps) composed of an initial transient hyperpolarization (fast component) followed by a slow recovery to resting potential (slow component).

3 L-Nitro-arginine-methyl ester (L-NAME, 100 μM) selectively abolished the slow component of i.j.ps. The effects of L-NAME were reversed by L-arginine (0.2–2 mM) but not by D-arginine (2 mM). Sodium nitroprusside (an NO donor, 1 μM) reversibly hyperpolarized muscle cells. This suggests that NO mediates the slow component of i.j.ps.

4 L-Citrulline (0.2 mM) also reversed the effects of L-NAME, and this action was maintained during sustained exposures to L-citrulline (0.2 mM). This may reflect intraneuronal recycling of L-citrulline to L-arginine.

5 Higher concentrations of L-citrulline (e.g. 2 mM) had time-dependent effects. Brief exposure (15 min) reversed the effects of L-NAME, but during longer exposures (30 min) the effects of L-NAME gradually returned. In the continued presence of L-citrulline, L-arginine (2 mM) readily restored nitrergic transmission, suggesting that during long exposures to high concentrations of L-citrulline, the ability to generate arginine from citrulline was reduced.

6 Aspartate (2 mM) had no effect on i.j.ps, the effects of L-NAME, or the actions of L-citrulline in the presence of L-NAME. L-Citrulline (0.2–2 mM) alone had no effect on i.j.ps under control conditions.

7 **S**-methyl-L-thiocitrulline (10 μM), a novel NOS inhibitor, blocked the slow component of i.j.ps. The effects of this inhibitor were reversed by L-arginine (2 mM), but not by L-citrulline (2 mM).

8 These results suggest that i.j.ps in the murine colon result from release of multiple inhibitory neurotransmitters. NO mediates a slow component of enteric inhibitory neurotransmission. Recycling of L-citrulline to L-arginine may sustain substrate concentrations in support of NO synthesis and this pathway may be inhibited when concentrations of L-citrulline are elevated.

Keywords: Citrulline; arginine; nitric oxide; enteric nervous system; argininosuccinate; colon; inhibitory junction potential; gastrointestinal motility

Introduction

Nitric oxide (NO) has been identified as a neurotransmitter utilized by inhibitory neurones innervating many gastrointestinal (GI) smooth muscles (see Rand & Li, 1995; Stark & Szurszewski, 1992; Sanders & Ward, 1992; Shuttleworth & Sanders, 1996, for reviews). The enzyme responsible for neuronal nitric oxide synthesis (nitric oxide synthase, NOS) catalyses the conversion of arginine to equimolar amounts of NO and citrulline (Bredt & Snyder, 1989). When strips of GI muscles are treated with arginine analogues that are competitive inhibitors of NOS, such as L-nitroarginine-methyl ester (L-NAME), NO-dependent (nitrergic) neurotransmission is reduced or abolished. Nitrergic transmission can then be restored in the continued presence of competitive NOS inhibitors by addition of excess L-arginine (eg Bult *et al*., 1990; Dalziel *et al*., 1991; Stark *et al*., 1991).

We recently provided evidence suggesting that the intraneuronal supply of arginine required for NO synthesis might be maintained by the recycling of citrulline within canine enteric neurones (Shuttleworth *et al*., 1995). Immunoreactivity for argininosuccinate synthetase (AS) and argininosuccinate lyase (AL) were selectively found in NOS-containing enteric neurones. These urea cycle enzymes convert citrulline to arginine via the production of argininosuccinate (Ratner, 1973). This suggests that nitrergic neurones express the enzymatic apparatus necessary to (1) synthesize NO and citrulline from arginine and (2) regenerate arginine from citrulline via argininosuccinate. Functional tests of this hypothesis were performed with L-citrulline to overcome selectively block of nitrergic neurotransmission by L-NAME (Shuttleworth *et al*., 1995). The expression of AS and AL in NOS-containing neurones has not yet been demonstrated in other regions of the autonomic nervous system, but similar pharmacological observations in studies of the murine anococcygeus (Gibson *et al*., 1990) and in guinea-pig and human bronchial muscles (Ellis & Conanan, 1994) suggest this pathway may be widely used to maintain nitrergic neurotransmission. Chen and Lee (1995) have recently shown conversion of [^{14}C]citrulline to [^{14}C]arginine in cerebral arteries and proposed this as a mechanism for maintaining substrate for NO synthesis in neurones supplying these vessels.

In the absence of potent and selective inhibitors of AS and AL activity, the hypothesis of citrulline-arginine recycling might be tested with transgenic mice that lack the gene coding for AS (Patejunas *et al*., 1994). The initial aim of the present study was therefore to determine whether murine GI tissues are suitable for studies of neuronal citrulline-arginine recycling. A component of inhibitory transmission due to NO was identified, and the ability of citrulline to restore NO synthesis after inhibition with arginine analogues was tested. In the course of these experiments, we observed an unusual concentration-dependent action of citrulline, suggesting that regulation of ci-

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trulline-arginine recycling occurs under conditions of high substrate load. Several possible mechanisms for this inhibition have been investigated. Finally, the actions of a novel NOS inhibitor, S-methyl-L-thiocitrulline (SMTC), were investigated on NO synthesis and citrulline actions in murine nitrergic transmission. A recent study has noted that SMTC block of nitrergic transmission in opossum oesophagus is reversed by arginine but not citrulline (Ledow *et al*., 1996), raising the possibility that SMTC inhibits recycling of citrulline to arginine. Some of these results have been presented in abstract form (Conlon *et al*., 1996).

Methods

General

Balb C mice (BALB/cAnN Sim) of either sex were obtained from Simonsen Laboratories (CA, U.S.A.). Animals were anaesthetized by chloroform inhalation and killed by cervical dislocation and decapitation, as approved by the University of Nevada Laboratory Animal Use and Care Committee. The abdomen was opened and a segment of proximal colon 20–30 mm from the ileocoecal sphincter removed. The colon was opened along the mesenteric border, washed of remaining faecal material and pinned out in a dissecting dish containing oxygenated Krebs-Ringer bicarbonate (KRB) solution. Mucosa and submucosa were removed, resulting in sheets of tissue comprising circular and longitudinal muscle layers, together with the attached myenteric plexus.

Intracellular electrical recording

Strips of muscle were pinned to the floor of an electrophysiological chamber, with the exposed circular muscle layer uppermost. Tissues were constantly perfused with warmed, oxygenated KRB maintained at $32 \pm 0.5^{\circ}$C. Muscle strips were allowed to equilibrate for 90–120 min before experiments were begun. Atropine and nifedipine (both 1 μM) were present throughout all experiments. Cells within the circular muscle layer were impaled with glass microelectrodes filled with 3 M KCl and having resistances of 30–50 MΩ. Impalements were accepted based on previously discussed criteria (Sanders & Smith, 1986). Membrane potential was measured with a high input impedance electrometer (WPI S-7100) and outputs were displayed on an oscilloscope (Tektronix 5111A). Analogue electrical signals were recorded on magnetic tape (Hewlett Packard 3964A) and reproduced on chart paper (Gould 2200). In some experiments, signals were also digitized (MP100A, Biopac Systems, CA) and recorded with a PC-based data acquisition system (Acknowledge version 3.03, Biopac Systems, CA).

Neurones were stimulated via platinum wire electrodes connected to a Grass S88 stimulator via a stimulus isolation unit (Grass SIU5). Single pulses (delivered at 0.03 Hz) or trains of stimuli (10 pulses delivered at 1–20 Hz, 0.5 ms duration, 15 V) produced transient hyperpolarizations of membrane potential (inhibitory junction potentials; i.j.ps) that were abolished by tetrodotoxin (1 μM).

Statistical analysis

All data are expressed in terms of mean $\pm$ s.e.mean. In all experiments individual tissues were exposed sequentially to a series of drugs and paired *t* tests were employed to test between treatments, comparing the matched values only from each tissue that received each drug. *n* values refer to the number of experiments performed in tissues from different animals.

Solutions and drugs

The KRB contained (in mM): NaCl 120.35, KCl 5.9, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 15.5, NaH_2PO_4 1.2 and dextrose 11.5. This solution had a pH of 7.4 at 32°C when bubbled to equilibrium with 97% O_2/3%CO_2. L-Arginine, D-arginine, L-citrulline, N^G-nitro-L-arginine methyl ester (L-NAME), atropine sulphate, nifedipine, were all obtained from Sigma Chemical Co. (St. Louis, MO). S-methyl-thiocitrulline was from Alexis Corp. (San Diego, CA). A stock solution of nifedipine (10 mM) was prepared in ethanol and all other drugs were dissolved in KRB.

Results

In all experiments nifedipine (1 μM) was added to the external solution to minimize muscle contractions, and atropine (1 μM) was included to antagonize cholinergic excitatory junction potentials. In the absence of other drugs or stimulation, circular smooth muscle cells displayed stable resting membrane potentials (RMP; mean -43.0 ± 1.5 mV in cells from 24 tissues); action potentials or spontaneous hyperpolarizations were not observed.

Control responses to electrical field stimulation

Electrical field stimulation (EFS) was used to stimulate neurones supplying the muscularis. Single pulses of EFS (0.5 ms, 15 V) produced transient hyperpolarizations (i.j.ps) that consisted of two distinct components (Figure 1). The initial component was characterized by a rapid hyperpolarization, 21.4 ± 0.6 mV amplitude ($n = 23$), that recovered to 76.4 $\pm 2.5\%$ ($n = 24$) of control RMP within 1 s following the stimulus. We have referred to this as the 'fast component' of the i.j.p. A second component was characterized by a distinct and gradual recovery to RMP, and we have termed this the 'slow component'. The duration of the slow component was measured as the time from the end of the stimulus to 90% recovery of RMP and this event averaged 4.9 ± 0.6 s in response to single pulse of EFS ($n = 24$). Both components of the i.j.p. were blocked by tetrodotoxin (TTX, 1 μM; $n = 3$).

Trains of EFS (10 pulses delivered at 1–20 Hz, 0.5 ms, 15 V) produced compound i.j.ps that were also composed of fast and slow components. During stimulus trains delivered at 1 Hz, each pulse produced a discrete hyperpolarization and a partial repolarization between pulses. Stimulation at higher frequencies (2–20 Hz) resulted in fusion of single i.j.ps and

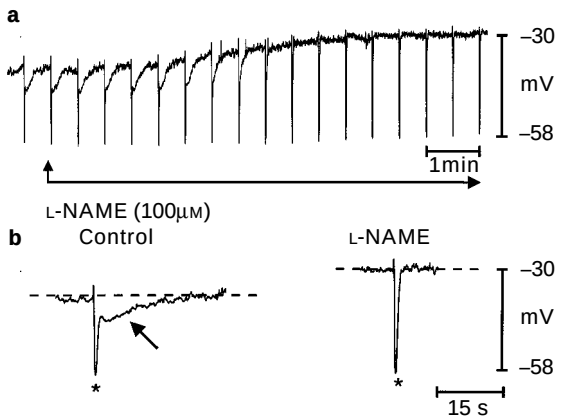

Figure 1 L-NAME selectively blocked a slow component of inhibitory junction potentials. All panels show intracellular microelectrode recordings of membrane potential from a circular smooth muscle cell, demonstrating transient hyperpolarizations (i.j.ps) evoked by single pulses of electrical field stimulation. (a) A series of i.j.ps evoked by continuous stimulation at 0.03 Hz, two of these events are reproduced in (b) at an expanded time base and the broken line indicates resting membrane potential. Under control conditions, i.j.ps consisted of two components, an initial rapid hyperpolarization (asterisk in (b)) followed by a sustained hyperpolarization (arrow in (b)). L-NAME (100 μM) selectively blocked the sustained component of i.j.ps and produced a small depolarization.

sustained hyperpolarization during the stimulus train. At stimulus frequencies 1–10 Hz, the maximum amplitude of the i.j.p. was reached during the stimulus train. Following a train of stimuli delivered at 20 Hz, the maxium hyperpolarization was reached approximately 0.5 s after completion of the train. The maximum amplitude of the compound i.j.p. was not significantly different at the stimulus frequencies tested (i.e. 21.9 ± 0.6 mV, 22 ± 0.6 mV, 22.5 ± 0.6 mV, 22.8 ± 0.8 mV and 23.5 ± 0.8 mV at 1, 2, 5, 10 and 20 Hz, respectively). Following trains of stimuli at all frequencies (1–20 Hz), there was a slow recovery to RMP following the train. The duration of this slow component did not appear to be frequency-dependent (e.g. the slow component averaged 5.4 ± 0.6, 6.2 ± 0.6, 6.2 ± 0.5, 6.6 ± 0.8, 5.3 ± 0.7 s at 1, 2, 5, 10 and 20 Hz, respectively; $n=24$). All phases of the compound i.j.ps in response to 1–20 Hz stimulation were blocked by TTX (1 μM, $n=3$).

Effects of L-NAME

The nitric oxide synthase inhibitor L-nitro-arginine-methyl ester (L-NAME, 100 μM) produced a significant depolarization of smooth muscle cells (RMP -43.0 ± 1.5 mV in control and -37.0 ± 1.0 mV in L-NAME $P<0.05$, $n=24$) (Figure 1). The amplitude of the fast component of i.j.ps increased in the presence of L-NAME (data not shown), probably due to an increase in driving force for K^+ ions caused by depolarization. In contrast, L-NAME abolished the slow component of i.j.ps (Figures 1 and 2, Table 1) at all stimulus frequencies tested. When single stimuli were tested, L-NAME selectively antagonized the sustained hyperpolarization seen after cessation of the stimulus (Figure 1). In addition, when short stimulus trains were used (e.g. <5 s, 2–20 Hz), L-NAME selectively antagonized the sustained hyperpolarization that occurred after cessation of the stimulus train (Figure 2b). When longer stimulus trains were used (e.g. 10–20 s trains at 1–5 Hz), the sustained hyperpolarization during the train was also reduced by L-NAME, suggesting that it takes time for the NO-dependent effects to develop (Figure 2a,c). In many preparations, a depolarization above RMP was observed following the slow component of i.j.ps. Under the conditions of these experiments, these depolarizations were typically reduced following L-NAME exposure (eg Figure 2).

Maximal effects of L-NAME occurred within 15 min of exposure to L-NAME. The actions of L-NAME were reversed by L-arginine, but not by D-arginine. Table 1 summarizes the effects of L-NAME and L- and D-arginine on the slow component of i.j.ps, measured as the time to recover to 90% of resting membrane potential following the completion of a single stimulus and a train of 10 pulses.

Actions of sodium nitroprusside

The effects of L-NAME suggest that endogenous NO contributes to hyperpolarization in response to nerve stimulation. Further evidence supporting this hypothesis comes from the actions of an NO donor, sodium nitroprusside (SNP). In the presence of L-NAME (100 μM), SNP (1 μM) produced hyperpolarization (20.3 ± 1.6 mV, $n=4$) that reversed slowly after SNP washout. I.j.ps evoked by single pulses of EFS were reduced in amplitude during SNP exposure and a small depolarization response followed each i.j.p. when tissues were hyperpolarized by SNP (Figure 3).

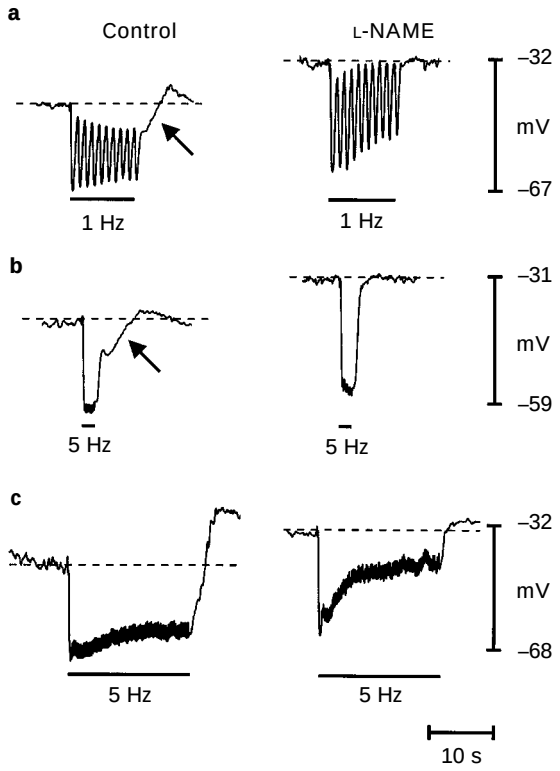

Figure 2 NO synthesis contributed to sustained hyperpolarization during and following trains of nerve stimulation. Left panels show control i.j.ps, (stimulus duration indicated by horizontal bars), and responses in the same cells following 20 min exposure to L-NAME (100 μM) are shown on the right. During stimulation at 1 Hz (a, 10 pulses) each pulse produced a discrete hyperpolarization. Under control conditions there was partial repolarization to resting potential between pulses, and a slow recovery to resting potential after cessation of the train (arrow). In L-NAME, cells almost completely recovered resting potential between each pulse, and immediately recovered resting potential after the stimulus train. (b) and (c) show that stimulation at a higher frequency (5 Hz) resulted in fusion of individual i.j.ps and a sustained hyperpolarization during the stimulus train. When the stimulus train was short (b, 10 pulses) L-NAME selectively antagonized the sustained hyperpolarization seen after cessation of the stimulus (arrow). Longer stimulus trains (c, 100 pulses), produced sustained hyperpolarization during the train which was also L-NAME-sensitive. Traces in (a) and (b) are excerpts from a continuous recording from one cell and traces in (c) are from a different tissue.

Table 1 L-NAME blocked the slow component of i.j.ps and these effects were reversed by L-arginine, but not D-arginine

	Control ($n=7$)	*L-NAME* (100 μM) ($n=7$)	*L-Arg* (0.2 mM) + *L-NAME* ($n=5$)	*L-Arg* (2 mM) + *L-NAME* ($n=6$)	*D-Arg* (2 mM) + *L-NAME* ($n=3$)
1 pulse	4.9 ± 0.9	1.2 ± 0.04*	1.6 ± 0.2	2.7 ± 0.4#	1.0 ± 0.1*
1 Hz	4.9 ± 0.5	1.2 ± 0.05*	3.4 ± 1.0	5.6 ± 0.9#	1.0 ± 0.1*
2 Hz	5.9 ± 0.6	0.9 ± 0.1*	5.1 ± 1.6	6.8 ± 0.6#	1.0 ± 0.1*
5 Hz	6.3 ± 0.8	1.3 ± 0.2*	5.5 ± 1.6	5.8 ± 1.6#	0.9 ± 0.1*
10 Hz	6.2 ± 0.6	1.8 ± 0.2*	4.8 ± 1.4	6.3 ± 1.5#	1.4 ± 0.7*
20 Hz	5.0 ± 0.7	2.2 ± 0.2*	3.7 ± 0.8	6.2 ± 1.5#	1.7 ± 0.1*

Values refer to the duration of the slow component of i.j.ps measured in seconds after completion of a single pulse or 10 pulse train. Effects of L-arginine and D-arginine were determined in different tissues. *$P<0.05$, compared with responses under control conditions; #$P<0.05$ compared with responses in L-NAME.

Reversal of L-NAME effects by L-citrulline

Similar to the actions of L-arginine, L-citrulline reversed the actions of L-NAME. The effects of L-citrulline were studied on tissues that were exposed to L-NAME (100 μM for at least 20 min) and repetitively stimulated at a frequency of 0.03 Hz. In the continued presence of L-NAME, exposure to 0.2 mM L-citrulline restored the sustained component of i.j.ps to the pre-L-NAME level at all stimulus frequencies tested (Table 2). The maximal effect was observed after approximately 20 min of exposure to L-citrulline. The ability of L-citrulline (0.2 mM) to restore the slow component of i.j.ps in the presence of L-NAME was sustained for periods in excess of 1 h. This is consistent with conversion of citrulline to arginine, which then competes with L-NAME for binding to NOS.

When higher concentrations of L-citrulline were used we noted that the effects of this compound were transient. For example, exposure to L-citrulline (2.0 mM) caused restoration

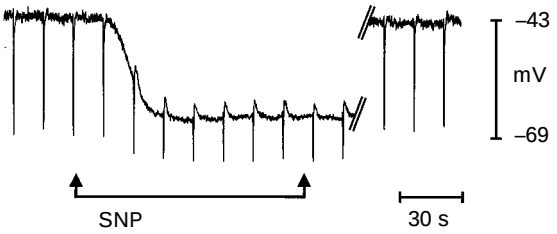

Figure 3 A nitric oxide donor (sodium nitroprusside, SNP, 1 μM) produced sustained hyperpolarization of resting membrane potential. L-NAME (100 μM) was present throughout and i.j.ps were evoked by continuous stimulation at 0.03 Hz. Effects of SNP were reversed by 25 min washout (indicated by break in trace).

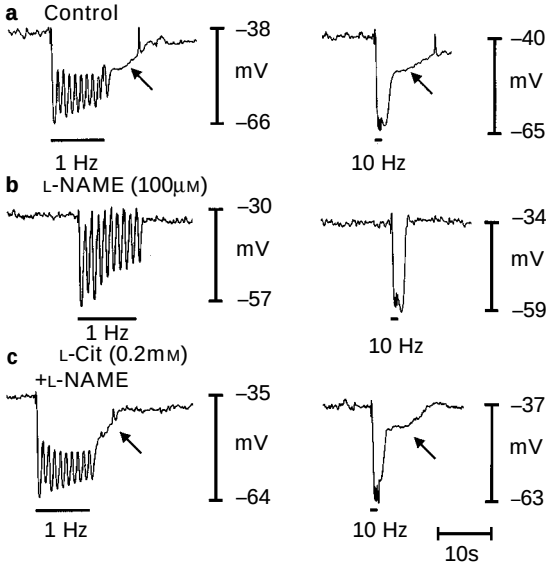

Figure 4 L-Citrulline reversed L-NAME block. (a) Control i.j.ps evoked by trains of 10 stimuli delivered at 1 Hz (left) and 10 Hz (right). The slow components of i.j.ps (arrows) were blocked by L-NAME exposure (b). In the continued presence of L-NAME, exposure to L-citrulline (0.2 mM, 20 min) reversed the L-NAME effects (c). All traces are excerpts from a continuous recording from a single cell.

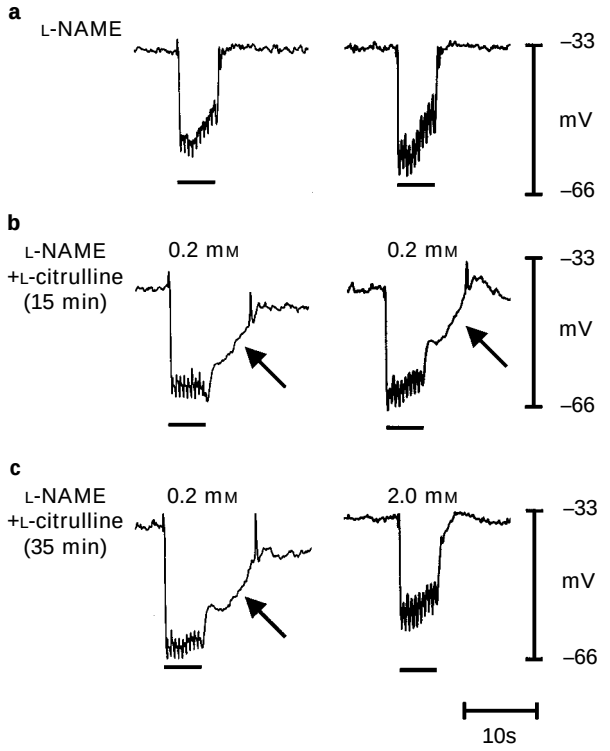

Figure 5 The effects of L-citrulline were concentration- and time-dependent. All panels show compound i.j.ps evoked by trains of stimuli (2 Hz, 10 pulses, indicated by bars). In the presence of L-NAME (100 μM, a) no slow component was seen following the stimulus train; 15 min after addition of L-citrulline (0.2 or 2.0 mM) the effects of L-NAME were reversed, a slow component to the i.j..p being revealed after the stimulus (b, arrows). The effects of 0.2 mM L-citrulline were maintained, such that after 35 min exposure, a prominent slow component was still evident (c, left panel, arrow). In contrast, after 35 min exposure to 2.0 mM L-citrulline, the slow component was virtually abolished (c, right panel), suggesting that antagonism of NO synthesis was re-established. Effects of 0.2 and 2.0 mM L-citrulline were investigated in different tissues, and each set of traces are excerpts from a continuous recording from a single cell.

Table 2 L-Citrulline (0.2 mM) reversed L-NAME actions, but this effect was lost following exposure to higher L-citrulline (2.0 mM) concentrations: subsequent L-arginine exposure restored the slow component of i.j.ps

	Control ($n=6$)	*L-NAME* ($n=6$)	*L-Cit* (0.2 mM) + *L-NAME* ($n=6$)	*L-Cit* (2 mM) + *L-NAME* ($n=5$)	*L-Arg* (2 mM) + *L-Cit* (2 mM) + *L-NAME* ($n=4$)
1 pulse	5.0±1.5	1.3±0.1*	3.3±0.7#	1.5±0.1#	4.7±1.1#
1 Hz	6.6±1.7	1.0±0.1*	9.5±2.6#	1.5±0.1#	8.5±0.8#
2 Hz	6.8±1.8	1.1±0.4*	9.9±1.7#	3.0±0.9	9.7±0.7#
5 Hz	5.5±1.0	1.0±0.1*	11.6±1.5#*	2.4±0.8	11.2±1.8#
10 Hz	4.9±0.9	1.3±0.1*	13.9±2.1#	2.7±0.8	10.5±4.1#
20 Hz	4.0±0.8	1.8±0.1*	11.1±2.7#*	2.3±0.2	9.8±4.6#

Values refer to the duration of the slow component of i.j.ps, measured in seconds after completion of a single pulse or 10 pulse train. *$P<0.05$ compared responses under control conditions; #$P<0.05$ compared with responses in L-NAME.

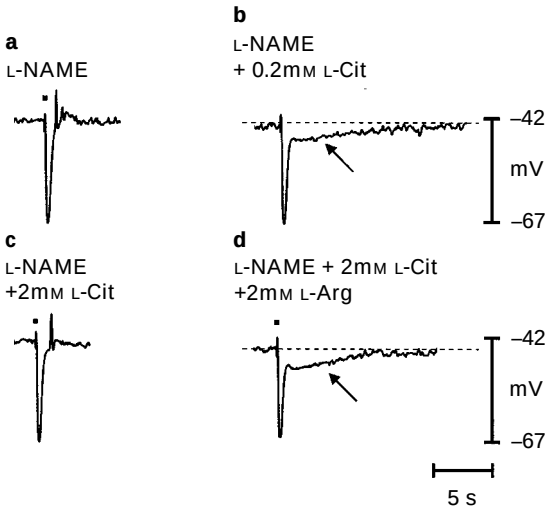

Figure 6 L-Arginine restores nitrergic transmission after tissues have become refractory to the actions of L-citrulline. All panels are excerpts from a continuous recording showing i.j.ps evoked by single pulses of EFS (0.03 Hz) in the presence of L-NAME (100 μM). Under control conditions (a), the i.j.p. comprised only a single component rapid hyperpolarization. Exposure to 0.2 mM L-citrulline (15 min) revealed a slow component to the i.j.p. (b, arrow). Subsequent addition of 2.0 mM L-citrulline blocked the sustained component of i.j.p. without affecting the initial component (c). Subsequent addition of arginine (2 mM) in the continued presence of (2mM) produced a full recovery of the sustained component (arrow). This suggests that tissues lost the ability to generate sufficient endogenous arginine for sustained competitive inhibition of L-NAME effects.

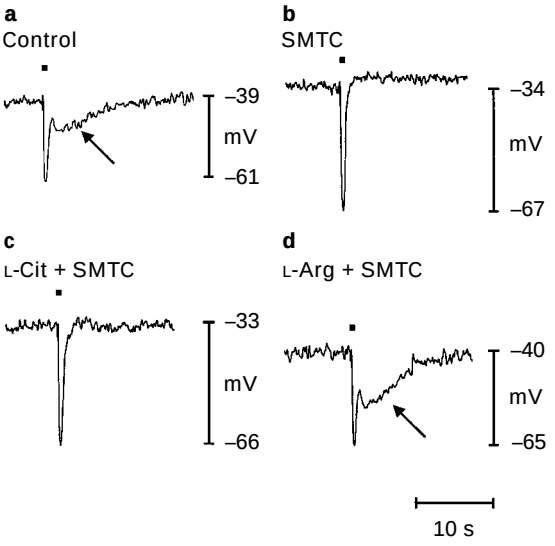

Figure 7 L-Arginine, but not L-citrulline, reversed the actions of S-methyl-thiocitrulline (SMTC). All panels show i.j.ps evoked by single pulses of EFS. The slow component of the i.j.p. (arrow) seen under control conditions (a) was blocked by SMTC (10 μM, b). Subsequent addition of L-citrulline (2.0 mM, 10 min), did not reverse the actions of SMTC (c), but addition of L-arginine in the continued presence of L-citrulline and SMTC restored the slow component of the i.j.p.

of i.j.ps in the presence of L-NAME within 15 min (repetitive stimulation at 0.03 Hz) and pre-L-NAME levels of the slow component were restored (Figure 3, Table 2). However, if the exposure to L-citrulline was continued, a progressive reduction in the slow component was noted such that this component was abolished after approximately 30 min (Figures 4 and 5; Table 2). Longer periods of exposure to L-citrulline produced no further reduction in i.j.ps, nor did this compound cause depolarization that might suggest deterioration of the impalement or preparation. In 4 control experiments the effects of L-citrulline (2 mM) were tested before L-NAME was added. In these tissues, long duration exposures to L-citrulline (2 mM, 1 h) had no effect on i.j.ps.

After tissues had become refractory to L-citrulline (e.g. 30 min exposure to L-citrulline in the continued presence of L-NAME) L-arginine (2 mM) rapidly and completely restored the slow component of i.j.ps at all frequencies tested (Figure 6, Table 2). This suggests that the ability of tissues to recycle L-citrulline to arginine was reduced during continued exposure to high concentrations of L-citrulline.

Effects of L-aspartate

The first step in the pathway proposed for citrulline recycling to arginine is condensation with aspartate to form argininosuccinate. The transient nature of L-citrulline effects, may have been due to depletion of aspartate. Therefore, tissues were pretreated with L-aspartate (2 mM) for 30 min before the addition of L-NAME and L-citrulline. L-Aspartate exposure alone had no effect on: (i) RMP or i.j.ps ($n=4$), (ii) the actions of L-NAME, (iii) the restoration of i.j.ps by L-citrulline (0.2 mM), or (iv) the transient restoration of i.j.ps by higher concentrations of L-citrulline (e.g. 2.0 mM).

Effects of S-methyl-thiocitrulline

The effects of L-NAME were compared with S-methyl-thiocitrulline (SMTC), a novel inhibitor of NOS (Furfine *et al*., 1994). Like L-NAME, SMTC (10 μM) selectively antagonized the slow component of i.j.ps evoked by single stimuli or trains of stimuli (Figure 7, Table 3). The effects of SMTC were readily reversed by L-arginine (2 mM) at all stimulus frequencies tested (0.03 – 5 Hz). In contrast, L-citrulline (0.2 – 2 mM) caused very little restoration of the slow component of i.j.ps in the presence of SMTC (Figure 7, Table 3).

Discussion

The circular muscle of murine proximal colon is supplied by enteric neurones containing NOS-like immunoreactivity (Sang & Young, 1996). Others have shown that neuronal release of NO regulates migrating myoelectric complexes in this tissue (Lyster *et al*., 1995). In the present study we have provided electrophysiological evidence that NO is responsible for a component of inhibitory transmission. An inhibitor of NOS activity (L-NAME) selectively blocked a slow component of inhibitory junction potentials (i.j.ps) at all stimulus frequencies tested, and these effects were reversed by L-arginine

Table 3 S-methyl-L-thiocitrulline (SMTC) blocked the slow component of i.j.ps: this effect was fully reversed by L-arginine, but not by L-citrulline

	Control	*SMTC* (10 μM)	*L-Cit* (0.2 mM) + *SMTC*	*L-Cit* (2 mM) + *SMTC*	*L-Arg* (2 mM) + *L-Cit* + *SMTC*
1 pulse	6.5 ± 0.7	1.0 ± 0.1*	1.5 ± 0.1*#	1.2 ± 0.1*	6.9 ± 1.1#
1 Hz	5.8 ± 0.7	0.8 ± 0.1*	1.1 ± 0.2*	1.3 ± 0.1*#	6.4 ± 0.3#
2 Hz	7.1 ± 0.9	1.0 ± 0.3*	1.8 ± 0.6*	1.5 ± 0.7*	8.2 ± 0.6#
5 Hz	8.2 ± 3.0	1.3 ± 0.1*	3.6 ± 1.2*	2.2 ± 0.8*	10.7 ± 2.4#

Values refer to the duration of the slow component of i.j.ps, measured in seconds after completion of a single pulse or 10 pulse train. *$P<0.05$ compared with responses under control conditions; #$P<0.05$ compared with responses in SMTC, $n=4$ for all values.

but not D-arginine. An NO donor (sodium nitroprusside) caused reversible hyperpolarization of the circular muscle. The finding that NO mediates only a relatively minor component of i.j.ps is similar to results from human colon (Keef *et al*., 1993) and jejunum (Stark *et al*., 1993). In those tissues NOS inhibitors were ineffective against the initial, fast component of i.j.ps, but reduced the sustained hyperpolarization resulting from longer stimulus trains. While the amplitude of NO-dependent effects are smaller than the fast component of i.j.ps, the sustained hyperpolarization attributed to NO release would result from increased K^+ conductance. This would reduce voltage-dependent Ca^{2+} channel open probability, reduce excitability and reduce Ca^{2+} entry into muscle cells. This scheme is consistent with data describing the contribution of NO-dependent neurotransmission on contractile activity of human colon (Boeckxstaens *et al*., 1993) and murine ileum (Goldhill *et al*., 1995).

The initial (or 'fast') component of i.j.ps was unaffected by L-NAME. The neurotransmitters responsible for this initial hyperpolarization are currently unknown, but may include transmitter(s) that activate an apamin-sensitive conductance (Lomax *et al*., 1996 and see Okasora *et al*., 1986). Candidate neurotransmitters for this fast component include ATP or a related purine (Hoyle & Burnstock, 1989) and pituitary adenylate cyclase activating peptide (PACAP) (McConalogue *et al*., 1995; and see Shuttleworth & Keef, 1995 for review). Enteric inhibitory neurones supplying GI smooth muscle contain markers for multiple inhibitory transmitters, and it appears that i.j.ps in many preparations are due to parallel release of NO and other transmitters from the same neurones.

In order to synthesize NO, enteric inhibitory neurones require a supply of L-arginine. Others have suggested that enteric glia maintain stores of L-arginine, and the amino acid could be supplied to neurones to support NO synthesis (Aoki *et al*., 1991). However, we recently presented evidence that NO-synthesising enteric neurones may not require an external supply of arginine, since NOS-containing neurones of the canine proximal colon have the enzymatic apparatus to convert L-citrulline to L-arginine. Thus, a system of citrulline recycling may serve to sustain NO-dependent neurotransmission. Functional studies support this hypothesis by demonstrating that L-citrulline could be used as a source of arginine in canine colon (see Introduction, Shuttleworth *et al*., 1995). However, it is noteworthy that the pharmacology of inhibitory transmission in canine proximal colon is quite distinct from many other gastrointestinal muscles, since all components of i.j.ps in that tissue are abolished by NOS inhibitors (Dalziel *et al*., 1991). As discussed above, in several other GI muscles, NO contributes to a small amplitude (but long duration) component of i.j.ps. In the present study, we have shown that L-citrulline is capable of restoring the NO-dependent component of i.j.ps in murine proximal colon, a tissue where NO produces one component of inhibitory responses. This suggests that murine enteric neurones have a functional citrulline-arginine cycle and indicates that the murine colon may be an appropriate tissue for investigations of citrulline recycling in transgenic mice in which elements of the pathway are knocked out (e.g. Patejunas *et al*., 1994).

A novel finding of this study is that the effects of citrulline were concentration- and time-dependent. At 0.2 mM L-citrulline had an arginine-like effect, causing sustained reversal of L-NAME inhibition of NO-dependent neurotransmission, but when higher concentrations were used, the effects of L-citrulline were transient. This phenomenon was not observed in canine proximal colon (Shuttleworth *et al*., 1995) and suggests some variability in the biochemical regulation of citrulline recycling between species. This observation has implications for the design of future studies to evaluate citrulline recycling because the concentration- and time-dependence observed in the present study might cause this pathway to be overlooked. This could lead to inaccurate conclusions about the importance of L-citrulline recycling in NO-dependent neurotransmission.

Some discussion should accompany the observation that the slow component of i.j.ps attributed to NO synthesis was not frequency-dependent over the range of stimulus frequencies tested in the present study. This seems in contrast to many studies in which marked frequency-dependence has been attributed to NO-dependent neurotransmission (e.g. Bult *et al*., 1990 and see Sanders & Ward, 1992, for review). In most studies in which responses have been characterized as a function of frequency the duration of stimulation has been held constant. Therefore, at the higher frequencies tested a far greater number of stimuli were applied than at low frequencies. In the present study we varied frequency while keeping the number of stimuli constant. Thus, a constant number of stimuli were delivered, but the period between stimuli was varied. The NO-dependent component of i.j.ps identified in mouse colon was slow enough that temporal summation appeared to be maximal over the entire range of frequencies tested. Since L-citrulline reversed the effects of L-NAME at all frequencies, this suggests that citrulline recycling is capable of maintaining arginine levels at relatively high frequencies of neural discharge.

The transient nature of L-citrulline effects is unlikely to be due to direct inhibitory effects of L-citrulline on NOS or the ion channels that mediate NO-dependent hyperpolarization that might occur as the compound builds up in cells, because long exposures to L-citrulline alone had no affect on NO-dependent neurotransmission in the absence of L-NAME. We also found that addition of L-arginine was able to reverse rapidly the effects of L-NAME on i.j.ps after the effects of L-citrulline had waned. These observations suggest that NO synthesis is not adversely affected by citrulline or a product of L-citrulline metabolism. It also appears unlikely that the time-dependent effects of L-citrulline resulted from depletion of aspartate, needed in citrulline recycling to form argininosuccinate, because supplementation with high concentrations of aspartate did not alter the transient nature of L-citrulline effects. Taken together, these experiments suggest that under conditions of high substrate load, murine enteric neurones lose the ability to convert L-citrulline to arginine. The mechanism of this regulation is not yet known, but it is possible that a byproduct of citrulline-arginine conversion causes feedback inhibition of this pathway.

The effects of SMTC are consistent with previous findings that this L-citrulline analogue acts as a NOS inhibitor (Furfine *et al*., 1994; Narayanan *et al*., 1995). SMTC blocked the slow component of i.j.ps and this effect was readily reversed by L-arginine. Ledow *et al*. (1996) recently found that the effects of L-citrulline did not reverse the effects of SMTC in opossum lower oesophageal sphincter muscle. A similar result was found in the present study, since L-citrulline only weakly reversed the actions of SMTC. It is possible that, in additon to NOS inhibition, SMTC antagonizes conversion of L-citrulline to L-arginine. This raises the possibility that SMTC inhibits NO production by a combination of two mechanisms, by (1) direct inhibition of NOS activity, and (2) limiting recycling of citrulline to arginine for NO synthesis. The relative importance of these two mechanisms is currently unknown. If analogues of citrulline can be found that block citrulline recycling without affecting NOS activity, these reagents would be extremely useful tools in determining the role of citrulline recycling in the maintenance of nitrergic neurotransmission.

In conclusion, L-citrulline, the co-product of neuronal NO synthesis, may be converted to L-arginine to maintain supply of substrate for NO synthesis. Our results provide functional evidence that a citrulline-arginine cycle is present in NO-synthesising neurones which supply the circular muscle of murine proximal colon. The effectiveness of this pathway appears limited under conditions of high substrate load, and it may also be inhibited by SMTC.

Note added in proof

Since submission of this study, two reports have been published describing an NO-dependent component of the inhibitory junction

potential in the murine colon. These responses are similar to the control responses described in the present paper. The reader is referred to: Watson, M.J., Bywater, R.A.R., Taylor, G.S. & Lang, R.J. (1996). Effects of nitric oxide (NO) and NO donors on the membrane conductance of circular smooth muscle cells of the guinea-pig proximal colon. *Br. J. Pharmacol.*,**118,** 1605 – 1614; and Watson, M.J., Lang, R.J., Bywater, R.A.R. & Taylor, G.S. (1996). Characterization of the membrane conductance changes underlying the apamin-resistant NANC inhibitory junction potential in the guinea-pig proximal and distal colon. *J. Auton. Nerv. Syst.*, **60,** 31 – 42.

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