Characterization of a putative nitric oxide synthase in the neuromuscular system of the parasitic nematode, *Ascaris suum*

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SUMMARY

In this paper we report on the biochemical presence of nitric oxide synthase (NOS)-like activity in *Ascaris suum* tissue and examine the pharmacological effect of NO donors on *A. suum* muscle strip preparation. NOS activity was determined by monitoring the formation of [^{3}H]citrulline from [^{3}H]L-arginine and NO formation via the oxyhaemoglobin assay. Neuromuscular tissue from *A. suum* which stained positively for NADPH diaphorase, contained NOS activity. Neither NOS activity nor NADPH diaphorase staining was detected in intestinal tissue. The absence of Ca^{2+}, NADPH and other co-factors normally required for mammalian neuronal NOS activity only partially reduced the formation of both citrulline and NO by *A. suum* neuromuscular homogenate. The results of the biochemical assays indicate the presence of an enzyme capable of producing NO and citrulline, but with a different profile from that of rat neuronal NOS. We also present preliminary evidence for the action of NO (NO donors) in the neuromuscular system of *A. suum*.

Key words: *Ascaris suum*, nematode, invertebrate, nitric oxide synthase.

INTRODUCTION

The nervous system of nematodes contains several neuroactive substances including the classical neurotransmitters acetylcholine (ACh), (γ-amino butyric acid (GABA) and 5-hydroxytryptamine (5-HT) (Walker, Brooks & Holden-Dye, 1996). In addition, a diverse range of neuropeptides including FMRF-amide like peptides have been localized immunocytochemically in the nervous system of nematodes (for review see Brownlee *et al*. 1996; Day & Maule, 1999).

Nitric oxide (NO) has been identified as a neuronal messenger, performing diverse signalling tasks in the central and peripheral nervous system of mammals (see reviews by Garthwaite & Boulton, 1995 and Rand & Li, 1995). NO and the co-product citrulline are generated from arginine in a reaction catalysed by nitric oxide synthase (NOS). NOS present in mammalian neurons is characterized by a dependency on specific co-factors, including nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), tetrahydro-L-biopterin (BH_4) (Mayer, John & Bohme, 1990), Ca^{2+} and calmodulin (Bredt & Snyder, 1990), and inhibition by analogues of L-arginine, e.g. N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-nitro-L-arginine (L-NNA) (Nathan, 1992).

A role for NO as a neurotransmitter or neuromodulator in certain invertebrates has been widely reported (see review by Colasanti & Venturini, 1998). In particular, NOS has been localized in the CNS of a number of invertebrates (Elphick, Green & O'Shea, 1993; Elphick *et al*. 1995; Korneev *et al*. 1998), indicating the existence of NO-cyclic GMP signalling pathway. Of particular interest to the current study are reports indicating the presence and a role for the nitric oxide system in nematodes. Kaiser, Geary & Williams (1998) showed the production of NO from the filarial parasites *Dirofilaria immitis* and *Brugia pahangi*, while Pfarr & Fuhrman (2000) reported on positive immunohistochemical staining for NOS in *Brugia malayi*. In addition, Bowman *et al*. (1995) described NOS activity in the hypodermis/cuticle fraction of *A. suum* and in extracts of whole *Panagrellus redivivus*, which was partially inhibited by arginine analogue inhibitors of NOS. The NOS activity in *P. redivivus* extract was also reported to be partially dependent on exogenous calmodulin and completely dependent on Ca^{2+} (Bowman *et al*. 1995). A physiological role for NOS activity in *A. suum* was supported by the demonstration that exogenously-generated NO relaxed pre-contracted muscle strips and by evidence

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that NO mediates the inhibitory effects of the peptide PF1 (Bowman *et al*. 1995). A recent report has also proposed a role for endogenously produced NO by *A. suum* in haemoglobin-mediated detoxification of oxygen (Minning *et al*. 1999).

We have reported on the distribution of NADPH diaphorase activity in the nervous system of the parasitic nematode *A. suum* (Bascal *et al*. 1995*a*). NADPH diaphorase has been reported to be responsible for neuronal NOS activity in mammals (Dawson *et al*. 1991), thus serving as a histochemical marker for the localization of NOS in the nervous system of fixed mammalian tissue (Hope *et al*. 1991). The specificity of this technique is attributed to the inactivation of other cellular NADPH diaphorases during formaldehyde fixation (Matsumoto *et al*. 1993). In addition, we reported on the neuronal co-localization of NADPH diaphorase with a SALM-Famide like peptide and with calcitonin gene-related peptide (CGRP) in *A. suum* (Bascal *et al*. 1996). These reports from various research groups, suggest the possible existence of nitrergic neurons and a neurotransmitter or neuromodulatory role for NO in nematodes, possibly linked to neuropeptide activity. However, while there is growing evidence for the presence of NOS in nematodes, it should be noted that the *C. elegans* genome project has not identified any sequences with homology to NOS enzymes and also that NO-sensitive guanylate cyclase and NOS enzyme activities were not detected in *C. elegans* (Hudson & O'Shea, 1998; Morton *et al*. 1999).

In the original report of the presence of NOS in *A. suum* and *P. redivivus*, Bowman *et al*. (1995) measured enzyme activity by monitoring the conversion of arginine to the co-product citrulline. They observed higher rates of NOS activity in extracts of whole *P. redivivus* compared with *A. suum*. This coupled with the greater availability of *P. redivivus* tissue, enabled them to perform a more detailed characterization of the *P. redivivus* enzyme than was possible using *A. suum* tissue. In this paper we report on further attempts to characterize NOS activity in *A. suum* tissue. We have monitored the production of both NOS catalysed reaction products, citrulline and NO, in tissues from *A. suum* and have determined the effect on this activity of cofactors and inhibitors which are specific for mammalian NOS. In addition, we examined the pharmacological effect of NO donors on *A. suum* muscle strip preparations.

Part of this work has been reported in abstract form (Bascal *et al*. 1995*b*) and presented at a symposium (Walker *et al*. 1995).

MATERIALS AND METHODS

Adult *A. suum* were removed from the small intestine of pigs obtained from local abattoirs. They were transported and maintained at 37 °C in artificial perienteric fluid (APF; composition in mM, NaCl 67, Na acetate 67, $MgCl_2$ 15·7, $CaCl_2$ 5, KCl 3, Tris 5, glucose 2, pH 7·6 with glacial acetic acid) for up to 5 days. Each *A. suum* was dissected open on a Sylgard plate containing APF. The intestine was gently removed. Both ventral and dorsal nerve cords were dissected out. The nerve cord tissue contained nerve cords as well as surrounding muscle cells and underlying hypodermis and cuticle. The dissected tissues were stored in liquid nitrogen until required.

Cerebella, dissected from adult female Wistar rats, were stored at −70 °C until used. This tissue, which is rich in NOS activity, was used as a positive control for the NOS assays (Bredt & Snyder, 1990).

Chemicals were supplied by Sigma and BDH unless stated otherwise.

Preparation of tissue extract

Frozen samples (e.g. *A. suum* nerve cords and intestines and rat cerebellum) were homogenized in ice-cold buffer composed of 50 mM Tris–HCl (pH 7·4), 0·5 mM EDTA, 0·5 mM EGTA, 20 μM leupeptin, 1 μM pepstatin A, 10 μM E-64, 40 μg/ml bestatin, 0·5 mM dithiothreitol (buffer A). The homogenate was centrifuged at 100000 ***g*** at 4 °C for 60 min (Bredt & Snyder, 1990). The supernatant (crude extract) was then used in the citrulline synthesis assay. In addition, some samples of crude extract were either filtered using a Centricon 30 (cut off, 10 kDa) microconcentrator (Amicon, Danvers, MA) or purified using 2′,5′ ADP-Sepharose. Purification involved the addition of tissue extract (300–500 μl) to 2′,5′ ADP-Sepharose (25 μl packed volume; Pharmacia) and mixing for 1 h at 4 °C. The mixture was pulse centrifuged and the supernatant removed. The pellet was washed successively (3 times in 500 μl for 20 min for each wash), by mixing and re-suspending as above, first with buffer A, then 0·5 M NaCl, and finally buffer A (minus EGTA, EDTA). Proteins were eluted from the ADP-Sepharose by mixing with 20 mM NADPH for 1 h at 4 °C.

To test for the presence of particulate NOS activity, a simple 2-step purification scheme was used (Forstermann *et al*. 1994). After homogenization and centrifugation of the tissue, the particulate fraction was washed for 10 min with 50 mM Tris-Cl (pH 7·4), 0·1 mM EDTA, 0·1 mM EGTA, 0·1 % mercaptoethanol, 10 % glycerol, 1 mM phenylmethylsulfonyl fluoride, 2 μM leupeptin, 1 μM pepstatin A, 1 M KCl (Buffer B) for 5 min. The mixture was centrifuged at 100000 ***g*** for 30 min. The resulting pellet was resuspended and solubilized for 20 min at 4 °C with 20 mM (3-[3-cholamidopropyl) dimethylammonia]-1-propane sulphonate (CHAPS) in buffer B. The mixture was then centrifuged again and the CHAPS extract was assayed for NOS activity using the citrulline synthesis assay.

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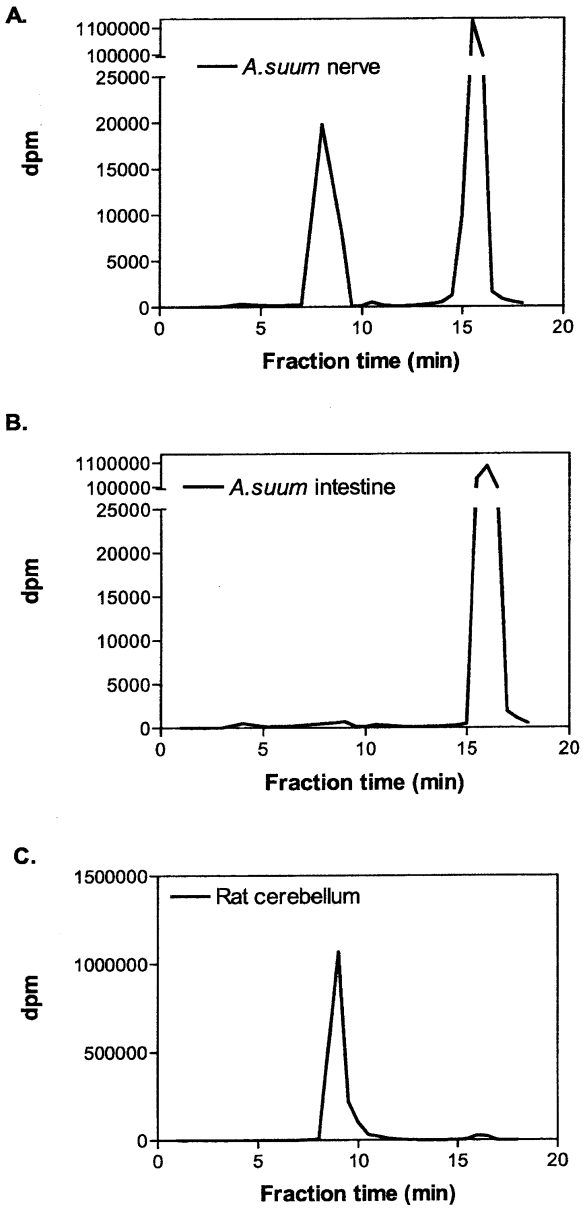

Fig. 1. HPLC separation of radio-isotope labelled substrate and products following incubation of [^{3}H]arginine with cytosolic extracts from (A) *Ascaris suum* nerve cord, (B) *A. suum* intestine, and (C) rat cerebellum. Fractions collected between 7 and 9 min co-elute with citrulline, while fractions collected 14–16 min correspond to arginine. Fractions co-eluting with citrulline were produced by rat cerebellum and *A. suum* nerve tissue, but absent from *A. suum* intestinal tissue.

Measurement of citrulline synthesis

NOS activity in soluble (crude extract, centricon-filtered, or ADP-Sepharose purified) and particulate fractions was estimated by measuring the conversion of L-[2,3,4,5,-^{3}H]arginine to L-[2,3,4,5,-^{3}H]citrulline using a method based on that of Bredt & Snyder (1990) and modified by Elphick *et al.* (1993, 1994). The reaction was performed by the incubation of 50 μl aliquots of tissue extract with an equal volume of an assay buffer containing; 2 mM NADPH, 2 mM Ca^{2+}, 20 μg/ml calmodulin, 20 μM BH_4, 10 μM FAD, 10 μM FMN, 50 mM valine (an arginase inhibitor), 40 μM arginine, and L-[2,3,4,5-^{3}H]arginine monohydrochloride (Amersham International plc) in 50 mM Tris–HCl (pH 7·4). After incubation for 60 min at 37 °C, the reaction was terminated either by the addition of 10 μl trifluoracetic acid (TFA; 50% v/v) or by the addition of 2 ml of ice-cold distilled water. The TFA-treated samples were boiled for 5 min and centrifuged to remove precipitated proteins. The supernatant was analysed using HPLC (Applied Biosystems Ltd, UK) combined with liquid scintillation counting to identify and quantify citrulline production (Cunningham & Rayne, 1997). Fractions containing [^{3}H]arginine, [^{3}H]citrulline and [^{3}H]ornithine were identified by comparison with the elution times of authentic radio-isotope labelled markers (L-[2,3,4,5-^{3}H]arginine monohydrochloride (Amersham International plc), L-[ureido-^{14}C]citrulline (NEN-Dupont), L-[U-^{14}C]-ornithine hydrochloride (Amersham, UK)). Elution times were typically 7–9 min (citrulline), 12–14 min (ornithine) and 14–16 min (arginine).

Reaction mixtures terminated with the addition of 2 ml of ice-cold distilled water were applied to cation exchange columns that retain arginine (1·5 ml Dowex AG 50W-X8 in a poly prepcolumn (Na^+ form)). The [^{3}H]citrulline activity was eluted in a total volume of 4 ml of distilled water and measured by scintillation counting, using LKB optiphase safe scintillant.

The identity of the reaction product was confirmed as citrulline by thin layer chromatography. Samples (10 μl) were loaded onto cellulose acetate sheets and separated using butanol:acetone:glacial acetic acid:water (35:35:10:20) as the mobile phase. The cellulose was cut into strips and radioactivity determined by scintillation counting. Separation of arginine and citrulline was confirmed using radio-isotope labelled authentic markers (RF 0.071 and 0.179 respectively).

Haemoglobin assay

NO synthesis was determined spectrophotometrically, using a modified version of the haemoglobin assay (Murphy & Noak, 1994) which is based on the reaction of NO and oxyhaemoglobin to form methaemoglobin. Tissue extract (0·25 ml) was added to a cuvette containing 0·25 ml of Tris–HCl buffer (50 mM, pH 7·4), catalase (100 U/ml), superoxide dismutase (SOD; 200 U/ml) and oxyhaemoglobin (8 μM). Catalase and superoxide dismutase were added to remove any potential interference by hydrogen peroxide and superoxide radicals respectively (Hevel & Marletta, 1994). The conversion of oxyhaemoglobin to methaemoglobin by NO was monitored (at 37 °C) by measuring the change in absorbance at 576 nm, using the isosbestic point with methaemoglobin (591 mm) as reference. The substrate arginine and the various co-factors:

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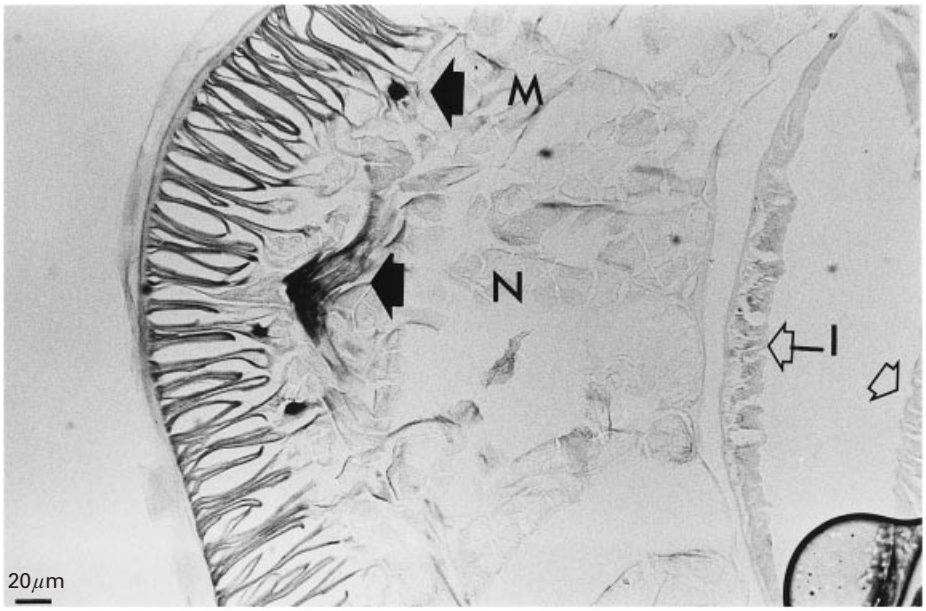

Fig. 2. NADPH diaphorase staining in transverse section of *Ascaris suum*. Positive staining is present only at selective sites; nerve cord (N) and muscle nuclei (M). Staining absent in the intestine (I).

NADPH, FAD, FMN, BH_4, calmodulin and Ca^{2+} were added in different orders depending on the experimental protocol followed. The rate of NO production was calculated by using the molar extinction coefficient of methaemoglobin at 576 nm (11·2 mM/cm).

Protein determination

Total protein concentration in tissue extracts was measured spectrophotometrically in 96-well plates using the Coomassie Plus protein assay reagent (Pierce Chemical) with bovine serum albumin as the standard.

In vitro *pharmacological studies*

The dorsal muscle strip preparation was made by cutting a 1 cm length of muscle tissue anterior to the genital pore of *A. suum*. This was cut lengthways along each lateral cord. The muscle preparation contains the dorsal muscle field, 1 or 2 cut commissures, the dorsal cord, lateral lines (1–2) and the terminals of dorsal inhibitory and dorsal excitor motorneurons, which innervate the muscle. The dorsal muscle strip was secured in an organ bath (volume 10 ml) and attached to an isometric transducer at a resting tension of 1 g in APF at 37 °C (Franks *et al*. 1994). The effects of ACh and various NO donors on the resting tension and phasic activity of dorsal muscle strip was investigated.

The NO donors used were hydroxylamine and acidified sodium nitrite. Stock solutions were prepared fresh each day, taking extreme care to minimize exposure to light and air. The stock solutions were kept cold and in the dark until just before use. They were diluted in APF and usually applied within minutes to the preparation.

Statistical analysis

All values are means ± S.E.M. of n experiments. Statistical significance was determined by Student's (paired and unpaired) t-test.

RESULTS

Citrulline synthesis

[^{3}H]citrulline production was detected using HPLC following incubation of a crude cytosolic extract of *A. suum* nerve cords with [^{3}H]-L-arginine in the presence of NADPH and the co-factors Ca^{2+}, calmodulin, BH_4, FAD and FMN (Fig. 1). The production of citrulline was tissue dependent and the distribution paralleled that of NADPH diaphorase staining. Thus no citrulline production was detected in *A. suum* intestinal tissue which also possessed no NADPH diaphorase staining, while nerve cords stained positively for NADPH diaphorase and produced a citrulline peak upon incubation with L-arginine (Figs 1 and 2). The rates of citrulline production by *A. suum* nerve cord and the positive control tissue, rat cerebellum, were 3·01 ± 0·5 pmol/min/mg protein (n = 5) and 269·7 ± 72·1 pmol/min/mg protein (n = 4) respectively. Preliminary studies also detected citrulline production in particulate preparations of *A. suum* nerve cord (5 pmol/min/mg protein), suggesting that both particulate and soluble

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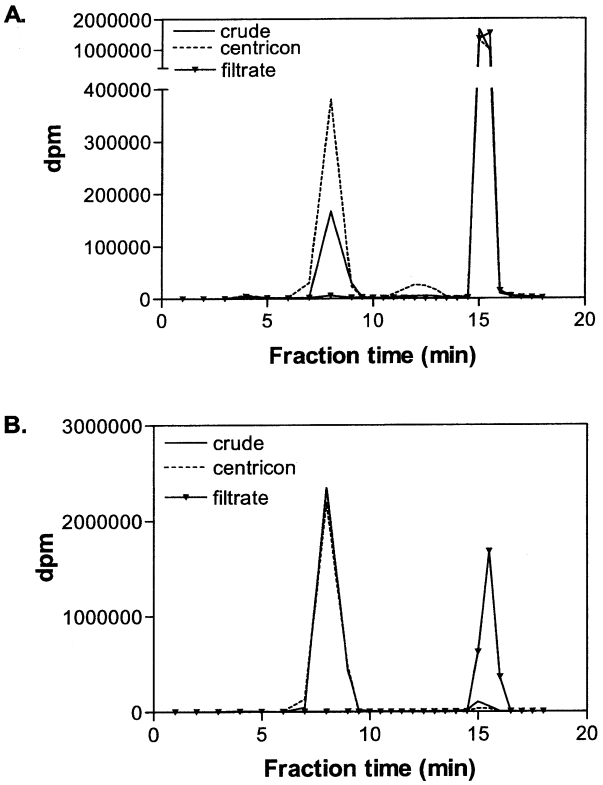

Fig. 3. HPLC separation of radio-isotope labelled substrate and products following incubation of [^{3}H]arginine with Centricon-filter separated extracts from (A) *Ascaris suum* nerve cord and (B) rat cerebellum. Extracts were crude (——) or separated using a Centricon filter (retained fraction; -----, filtered fraction; ▼). Activity was obtained in both crude and Centricon-retained fractions, but absent from filtrate fractions, indicating that the activity is due to a molecular entity larger than 10 kDa.

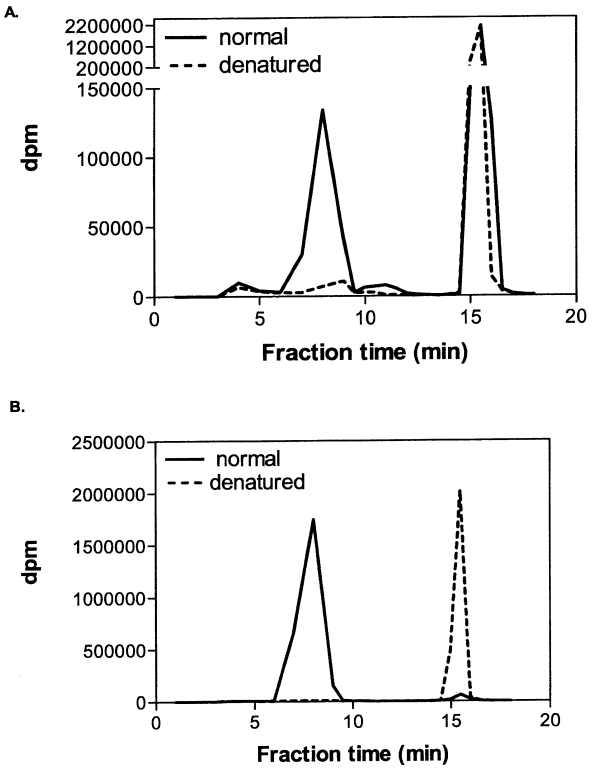

Fig. 4. HPLC separation of radio-isotope labelled substrate and products following incubation of [^{3}H]arginine with cytosolic extracts from (A) *Ascaris suum* nerve cord and (B) rat cerebellum. Extracts were untreated (normal; ——) or heat-denatured (-----). Conversion of arginine to citrulline was abolished in denatured samples for both species.

NOS activity exist. The synthesis of a radio-isotope labelled product from arginine by an *A. suum* crude extract, which co-eluted with citrulline was confirmed by TLC (data not shown).

Citrulline formation by both rat cerebellum and *A. suum* nerve cord was dependent on a large (> 10 kDa) molecular weight component as determined by retention on Centricon filters (Fig. 3). The tissue component responsible for formation of citrulline from arginine was heat sensitive since boiling tissue extract prior to assay completely eliminated citrulline production (Fig. 4).

In order to further characterize the putative NOS activity, extracts from *A. suum* nerve cord were incubated in a reaction mixture deficient in NADPH, Ca^{2+}, FAD, FMN, BH_4 and containing a NOS inhibitor (either L-NMMA, L-NNA or L-NAME; 1 mM) and the calmodulin antagonist *N*-(4-aminobutyl)-5-chloro-2-naphthalene-sulphonamide (W-13, 1 mM). This significantly ($P < 0{\cdot}05$) reduced the formation of citrulline by a crude extract of *A. suum* nerve cord to 69 % of the control (S.E.M. ± 3·5 %, $n = 7$) (Figs 5 and 6). The extent of inhibition was less than that observed by a preparation of crude extract from rat cerebellum (reduced to 0·4 %, S.E.M. ± 0·13 %, $n = 7$, $P < 0{\cdot}01$) (Figs 5 and 6). This significant reduction in activity of *A. suum* NOS could not be attributed to the presence of the NOS substrate antagonists (Table 1). By contrast, all or most of the inhibition of rat cerebellum NOS activity could be attributed to the presence of an arginine analogue inhibitor alone. Preliminary experiments were performed to assess the effect of other known NOS inhibitors, in addition to L-NNA, L-NAME and L-NMMA, on the putative NOS activity in *A. suum* nerve cord extracts. The addition of 1 mM of S-methylisothiourea, N^G-dimethyl-L-arginine (ADMA), or L-N^G (1-iminoethyl) lysine (NIO) to incubations of rat cerebellum crude extract, containing all added co-factors, reduced citrulline formation by 91–94 %. There was no indication of a similar potent effect of these inhibitors on citrulline production by crude extracts from *A. suum* nerve cord (citrulline production reduced by 16 %, 12 % and 3 % of control for S-methylisothiourea, ADMA and NIO respectively – data not shown).

To examine further the role of Ca^{2+}-calmodulin on the activity of the putative NOS activity in *A. suum*, the chelating agents EDTA and EGTA were used. In the absence of added NADPH, Ca^{2+}, FAD,

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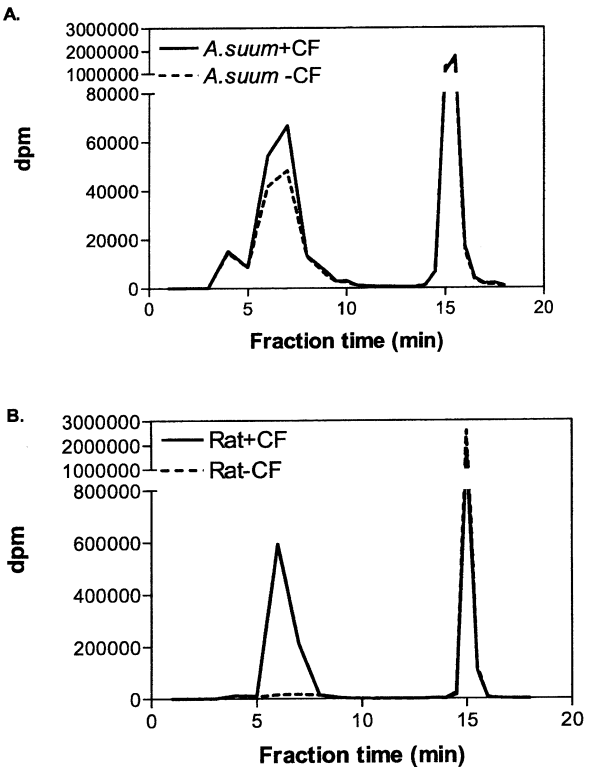

Fig. 5. HPLC separation of radio-isotope labelled substrate and products following incubation of [^{3}H]arginine with cytosolic extracts from (A) *Ascaris suum* (B) rat cerebellum. Extracts were incubated in the presence (+CF, ——) and absence (−CF, -----) of co-factors. The +CF assay buffer contained NADPH, Ca^{2+}, FMN, FAD, BH_4, calmodulin while the −CF assay buffer lacked these co-factors and, in addition, contained the calmodulin antagonist W-13 and the NOS inhibitor, L-NMMA. The removal of the co-factors reduced citrulline production by both *A. suum* nerve cord and rat cerebellum extracts.

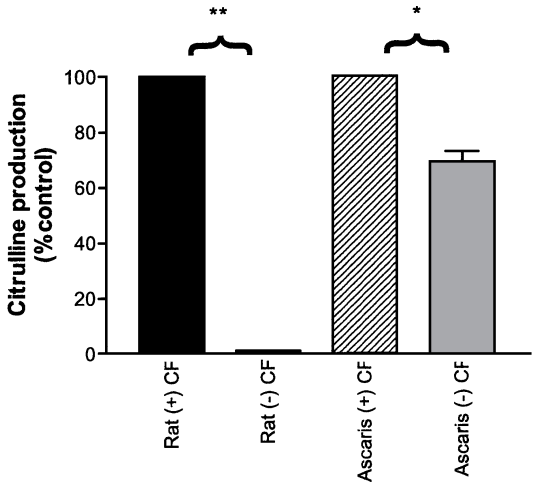

Fig. 6. The effect of co-factors on the conversion of arginine to citrulline by *Ascaris suum* nerve cord and rat cerebellum. The conversion of radio-isotope labelled arginine to citrulline by tissue extracts incubated in the presence (+CF) and absence (−CF) of co-factor was determined as described for Fig. 5. The quantity of citrulline produced in the −CF incubation is expressed as a percentage of that produced in the +CF incubation. The data are means ± S.E.M. for $n = 7$ (separate experiments). $^*P < 0{\cdot}05$ and $^{**}P < 0{\cdot}01$ compared to respective controls (using non-normalized values and Student's paired *t*-test).

FMN, BH_4, and calmodulin, 1 mM of EDTA and EGTA, reduced citrulline production by *A. suum* tissue by 31% (S.E.M. ± 5%, $n = 4$, $P < 0{\cdot}01$). However, higher concentrations of these chelating agents (25 mM EDTA, 12·5 mM EGTA) had the opposite expected effect, i.e. significantly ($P < 0{\cdot}05$) increasing citrulline production by *A. suum* nerve cord by 193% (S.E.M. ± 44%, $n = 4$) (Figs 7 and 8).

The differences in co-factor dependency between NOS isolated from *A. suum* and rat cerebellum could be due to the presence in the unpurified *A. suum* neuromuscular tissue of endogenous co-factors at concentrations sufficient to support enzyme activity when these were omitted from the assay reaction mixture. Similarly, high concentrations of endogenous arginine substrate in unpurified extract from *A. suum* might counter the competitive inhibitory effect of the arginine analogue used. Accordingly, attempts were made to purify *A. suum* NOS-like activity from the other tissue components using 2′,5′ ADP-Sepharose. However, while successful in purifying enzyme from rat cerebellum, this treatment resulted in a complete loss of NOS-like activity in crude extracts from *A. suum* (data not shown).

Table 1. The effect of arginine analogue inhibitors (L-NNA, L-NAME, L-NMMA) on the rate of arginine to citrulline conversion by extracts from *Ascaris suum* and rat cerebellum

(Extracts were incubated in the presence of all added co-factors (NADPH, calcium calmodulin, FAD, FMN, BH_4) (+CF) and in the presence of all added co-factors plus 1 mM L-NNA (+CF+L-NNA), L-NAME (+CF+L-NAME) and L-NMMA (+CF+L-NMMA). Values are expressed as % of arginine converted to citrulline during a 1 h incubation (mean ± S.E.M., $n = 2$–7 determinations) ($^*P < 0{\cdot}05$ compared with +CF value).

Condition	*Ascaris suum*	Rat cerebellum
+CF	6·8 ± 0·3	84·0 ± 3·9
+CF+L-NNA	6·0 ± 0·5	1·7 ± 0·6*
+CF+L-NAME	5·7 ± 0·1	1·3 ± 0·1*
+CF+L-NMMA	6·3 ± 0·1	2·0 ± 0·1*

Oxyhaemoglobin assay

The incubation of *A. suum* nerve cord extract with oxyhaemoglobin, resulted in the formation of methaemoglobin, suggesting the formation of NO (10·18 ± 3·7 pmol NO/min/mg protein, $n = 3$). The rate of conversion was increased by the addition of

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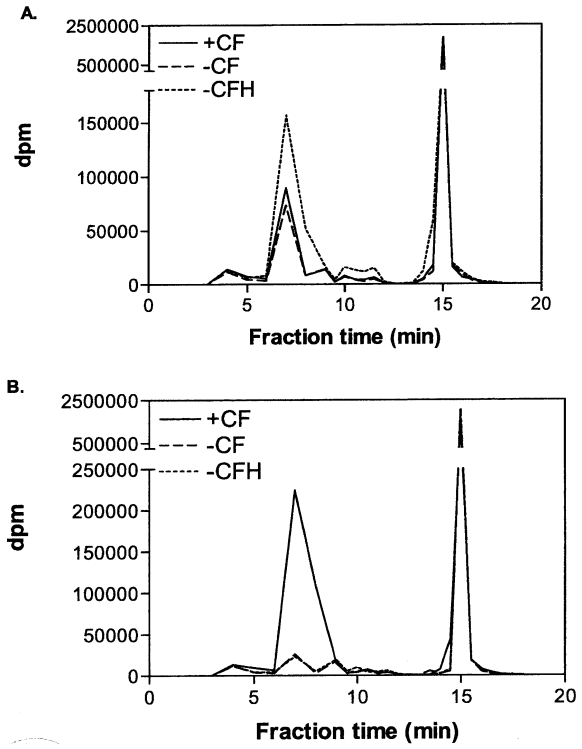

Fig. 7. The effect of EGTA and EDTA on citrulline production by (A) *Ascaris suum* nerve cord and (B) rat cerebellar crude extract. The extracts were incubated with co-factors (——, +CF), or without co-factors (-----, −CF) or without co-factors plus EDTA (25 mM) and EGTA (12·5 mM) (-----, −CFH).

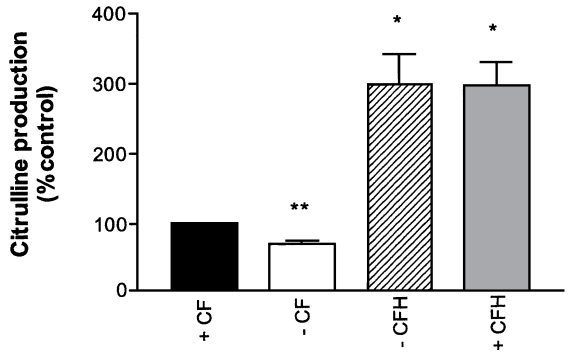

Fig. 8. The effect of EGTA and EDTA on citrulline production by *Ascaris suum* nerve cord crude extract. The extracts were incubated with co-factors (Ca^{2+}, calmodulin, NADPH, FAD, FMN, BH_4) (+CF), without cofactors (−CF), with co-factors plus EDTA (25 mM) and EGTA (12·5 mM) (+CFH) and without co-factors plus EDTA (25 mM) and EGTA (12·5 mM) (−CFH). Data expressed as a percentage of the enzyme activity detected under control conditions (presence of co-factors – see Materials and Methods section). Removal of co-factors (−CF) reduced citrulline production ($P < 0{\cdot}01$). The addition of high levels of chelating agents; EDTA (25 mM) and EGTA (12·5 mM), significantly ($P < 0{\cdot}05$) increased citrulline production in comparison to control (+CF) whether co-factors were present (+CFH) or absent (−CFH). The data are means ± S.E.M. for $n = 4$, $*P < 0{\cdot}05$, $**P < 0{\cdot}01$ (using non-normalized values and Student's paired *t*-test).

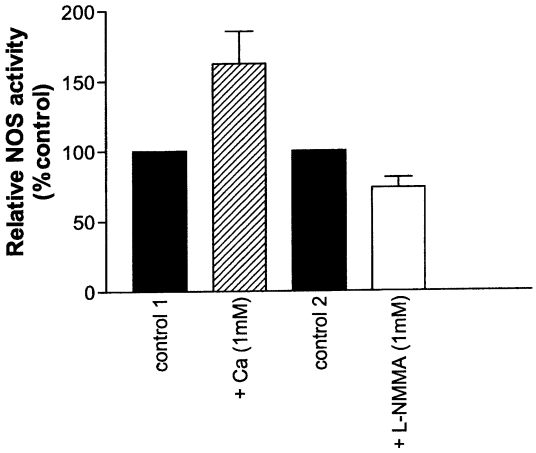

Fig. 9. Effect of Ca^{2+} and the NOS inhibitor L-NMMA on NO production by extracts from *Ascaris suum* nerve cord, using the oxyhaemoglobin assay. The two controls are different. Control 1, contains all co-factors, excluding Ca^{2+}. Control 2 contains all co-factors. The data for each set of experiments are expressed as a percentage of the enzyme activity detected under respective control conditions. Values are ± S.E.M. for $n = 3$–4. $P < 0{\cdot}1$ *vs* corresponding control (using non-normalized values and paired *t*-test).

1 mM Ca^{2+} (to 162 % of control, S.E.M. ± 23 %, $n = 4$, $P < 0{\cdot}1$) and reduced by the addition of the NOS inhibitor L-NMMA (to 73·5 % of control (containing Ca^{2+}), S.E.M. ± 7·2 %, $n = 3$, $P < 0{\cdot}1$) (Fig. 9). As a comparison, rat cerebellum produced 350·3 ± 103·8 pmol NO/min/mg protein ($n = 5$).

In vitro *pharmacological studies*

Preliminary experiments have been performed to test for the possible effect of exogenous NO on basal muscle tension and on ACh elicited contractions, using the NO donors; acidified sodium nitrite and hydroxylamine (0·1–10 mM). During rest, the muscle strip was stable (occasionally showing some rhythmic activity). The addition of ACh (1–100 μM) elicited a concentration-dependent muscular contraction. The addition of the NO donors (up to 10 mM) resulted in an increase in basal muscle tension, which was slow in onset. The effects were only observed with high concentrations of these weak NO donors (Fig. 10). In addition to this effect, the NO donors also reduced the size of contractions elicited by 10 μM ACh in a concentration dependent manner (Figs 10 and 11). The effects with 10 mM NO donor were significant ($P < 0{\cdot}05$ for nitrite, $n = 4$, $P < 0{\cdot}02$, for hydroxylamine, ($n = 5$) (Fig. 11). The effects were reversible upon washing suggesting a non-toxic effect of the NO donors on the tissue (Fig. 11).

DISCUSSION

We have previously reported on the distribution of NADPH diaphorase activity (a possible marker for NOS) and its co-localization with a SALMFamide-

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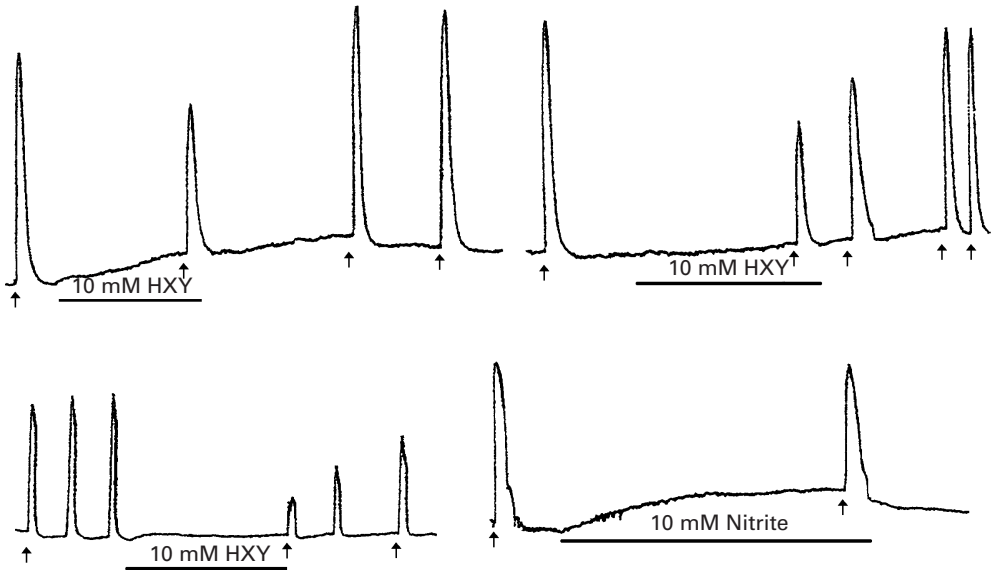

Fig. 10. *Ascaris suum* muscle tension recordings from 4 isolated *in vitro* dorsal muscle strip preparations, showing the effects of the NO donors hydroxylamine (HXY) and nitrite on basal muscle tension and ACh-induced muscle contraction. Muscle strips contracted in a readily reversible and reproducible manner to ACh. The arrows indicate the application of 10 μM ACh. The addition of the NO donors increased basal muscle tension (varied from tissue to tissue). The bars indicate the duration of application of the drugs. In the presence of NO donor, the contractions elicited by ACh were reduced. After a wash of 10 min or more the response to ACh was recovered. Similar results were obtained in further experiments.

like peptide and with CGRP, within certain neurons in the nervous system of the nematode *A. suum* (Bascal *et al*. 1995*a*, 1996). This together with the report of Bowman *et al*. (1995) of arginine to citrulline conversion by extracts of *A. suum* hypodermis/cuticle, suggests the possible existence of nitregeric neurons and a neurotransmitter or neuromodulatory role for NO in *A. suum*. In the study of Bowman *et al*. (1995) the putative NOS enzyme in *A. suum* was shown to be partially sensitive to known inhibitors of NOS. However, no further characterization of the enzyme was carried out due to the low enzyme activity and low availability of *A. suum* tissue. The aim of the present study was to further confirm the presence and role for a NOS-like enzyme within *A. suum* neuromuscular system using biochemical and functional studies. Accordingly, we have examined the ability of *A. suum* neuromuscular tissue to catalyse the formation of NO and L-citrulline from L-arginine under defined conditions. Citrulline was determined by monitoring the formation of [^{3}H]citrulline from [^{3}H]arginine. In addition NO formation was monitored using an assay based on the conversion of oxyhaemoglobin to methaemoglobin. Both assays were based on well established mammalian (rat) assays and rat cerebellum was used in parallel to *A. suum*, to monitor the reliability of the assays.

[^{3}H]citrulline formation was monitored using either HPLC or Dowex cation exchange chromatography. In situations where it is possible that other pathways of arginine metabolism are present and the products (e.g. ornithine, urea, polyamines, agmatine, creatinine) formed may fail to be separated from arginine, from each other or from citrulline, then HPLC is employed. For example, the enzyme arginase, which is prevalent in invertebrates, and has been detected in nematodes (see Barrett, 1981), can hydrolyse arginine to ornithine and urea. The HPLC method employed here can distinguish ornithine from arginine and urea from citrulline. In our experiments, only 2 major peaks were observed; a peak that co-eluted with arginine and another that co-eluted with citrulline. The arginine product was confirmed to be citrulline using TLC. No ornithine was detected using either HPLC or TLC. Dowex cation-exchange chromatography was used to monitor NOS activity only once it had been established by HPLC and TLC that citrulline alone was metabolized from arginine.

Our data indicate that the component of *A. suum* tissue which is capable of producing citrulline from arginine is heat sensitive and is greater than 10 kDa in size, properties which suggest an enzymatic protein. The enzyme activity is present in *A. suum* neuromuscular tissue, but absent from intestinal tissue. This agrees well with the distribution of NADPH diaphorase staining in fixed tissue, the classical NOS marker used in mammalian systems. Thus both biochemical and histochemical studies indicate that NOS is present in the neuromuscular system. The rate of arginine-derived citrulline production in *A. suum* tissue found in this study ($3{\cdot}01 \pm 0{\cdot}5$ pmol/min/mg protein) compares well with that obtained by other researchers using other invertebrates. For example, Elofsson *et al*. (1993)

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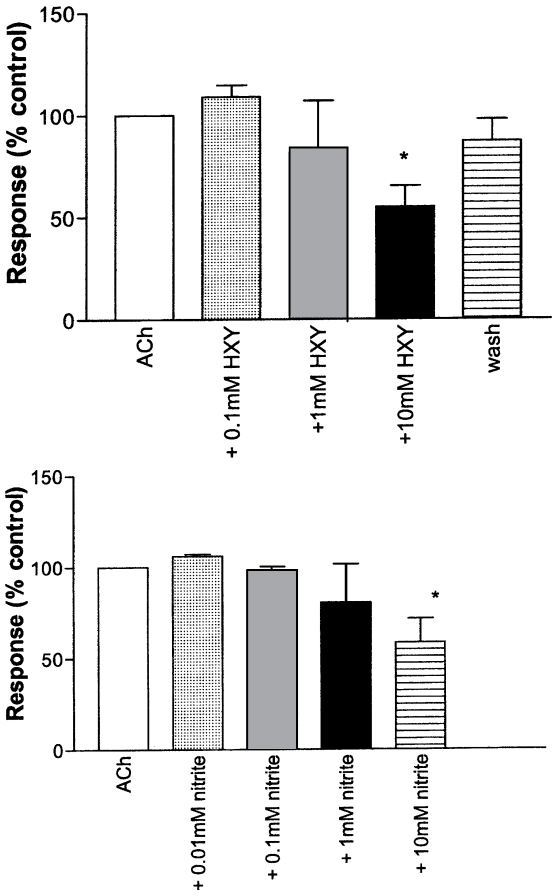

Fig. 11. Graphs showing the effect of 2 NO donors; hydroxylamine and sodium nitrite on contractions elicited by 10 μM ACh. The donors reduced the responses of ACh in a dose-dependent manner. The effects with 10 mM NO donor were significant ($P < 0{\cdot}05$ for nitrite, $P < 0{\cdot}02$ for hydroxylamine). The data are standardized as a percentage of response elicited by 10 μM ACh and expressed as mean $\pm$S.E.M. The non-normalized data were used for statistical analysis (Student's paired *t*-test).

reported the presence of 5·8 pmol/min/mg in CNS, and 14 pmol/min/mg in buccal ganglia of molluscs, while Bowman *et al*. (1995), obtained 0·47 pmol/min/mg and 0·19 pmol/min/mg from whole *P. redivivus* and *A. suum* hypodermis tissue respectively and Eriksson (1996) found 0·218 pmol/min/mg in whole planarian *Dugesia tigrina*.

The rate of arginine conversion to citrulline by *A. suum* tissue was far less than that produced by rat cerebellum homogenate (269·7 $\pm$ 72·1 pmol/min/mg). This value for rat cerebellum NOS activity was similar to that reported previously by other authors; 160 pmol/min/mg (Bredt & Snyder, 1990) and 338 pmol/min/mg (Rogers & Ignarro, 1992). In contrast to rat cerebellum, the *A. suum* tissue was not purely neuronal, but contaminated with a large proportion of muscular tissue, which would give rise to lower specific activity for *A. suum* 'neuronal' NOS. For a more accurate specific activity determination, individual nerve cords totally lacking in muscle tissue would be needed. However, due to the unique anatomical arrangement between muscle and nerve tissue of nematodes (Rosenbluth, 1965), the dissection is not feasible. Alternatively, since we have based our assay on a standard mammalian NOS assay, it is possible that it is inappropriate for *A. suum*, and has given rise to an underestimation of enzyme activity. Thus, absence or depletion of, as yet unidentified, cofactors or the presence of endogenous inhibitors of *A. suum* NOS may have rendered our assay system suboptimal for determination of *A. suum* enzyme activity.

In addition to confirming the conversion of arginine to citrulline, we demonstrated the conversion of oxyhaemoglobin to methaemoglobin, by *A. suum* tissue extract, thus further suggesting the production of NO. The rates of citrulline production and NO formation by rat cerebellum NOS were not significantly different from each other (269·7 $\pm$ 72·1 versus 350·3 $\pm$ 103·8 pmol/min/mg protein $P > 0{\cdot}05$ by Student's unpaired *t*-test) indicating stoichiometric generation of the co-products. However, there was a significantly lower rate of generation of citrulline versus NO by *A. suum* tissue (3·01 $\pm$ 0·5 versus 10·18 $\pm$ 3·7 pmol/min/mg protein). It is possible that the quantities of NOS products detected are affected by a non-stoichiometric production of NO which has a relatively greater effect on the NO/citrulline balance in *A. suum* compared with rat cerebellum NOS. This may arise from the presence of SOD in the haemoglobin assay mixture. Recently, Hobbs, Fukoto & Ignarro (1994) reported that SOD caused a marked concentration-dependent increase in the production of free NO by mechanisms that were unrelated to dismutation of superoxide anion or activation of NOS. SOD appears to elicit a novel biological action, perhaps accelerating the conversion of an intermediate in the L-arginine NO pathway such as nitroxyl (HNO) to NO. They also observed that whereas SOD enhanced the production of free NO in reaction mixtures containing NOS, SOD did not alter the formation of L-citrulline. This could help explain the non-stoichiometric relationship between citrulline and NO production observed with *A. suum* tissue in the present study. The situation may be potentiated by the fact that Cu–Zn SODs are abundant in tissues with high metabolic activity in helminths; in muscles and nerve trunks (see James, 1994).

To determine the co-factor requirements of the *A. suum* NOS-like enzyme, experiments were conducted in which complete enzyme reaction mixtures were compared with reaction mixtures deficient in Ca^{2+}, NADPH, FAD, FMN, BH_4, and containing a calmodulin antagonist and a NOS inhibitor. The elimination of the co-factors and the addition of NOS inhibitors and a calmodulin antagonist, led to only a partial reduction of *A. suum* NOS-like enzyme activity. This inhibitory effect could not be ac-

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counted for by the presence of the arginine analogue NOS inhibitors L-NAME, L-NNA or L-NMMA at a concentration of 1 mM. By contrast, Bowman *et al.* (1995) found that 100 μm, L-NNA and L-NMMA reduced the conversion of arginine to citrulline by preparations of *A. suum* hypodermis/cuticle by $\geqslant 50\,\%$. These workers pre-treated crude extracts of *A. suum* tissue with Dowex AG50W-XG resin to remove endogenous arginine. Consequently, it cannot be excluded that the difference between our results and those of Bowman *et al.* (1995) is due to the presence in our assayed extracts of high concentrations of endogenous arginine substrate, which counteracts the competitive inhibitory effect of the arginine analogue.

The partial reduction in enzyme activity upon the elimination of co-factors, suggests that either the crude extract was saturated with the endogenous cofactors, and therefore the effect of elimination from the enzyme mixture would be incomplete, or that more than one isoenzyme of NOS is present in *A. suum* neuromuscular tissue, and these differ by the nature of the co-factors required for activity. As regards the latter point, there may be 2 constitutive isoenzymes, one Ca^{2+} and calmodulin-dependent and the other Ca^{2+}-independent. The presence of more than one isoenzyme in the crude extract is possible, since NADPH diaphorase was detected in certain neurons and in the finger-like projections of the muscle arms (Bascal *et al.* 1995*a*), both of which would be present in our tissue extract. Alternatively, more than one isoenzyme may be present within neuronal tissue. There are several examples in the literature of constitutive neuronal NOS isoenzymes which are either partially dependent or independent of Ca^{2+} or calmodulin, in particular in invertebrate nervous tissue e.g. *Aplysia* ganglia (Moroz *et al.* 1996) and pharynx of the planarian flatworm, *Dugesia tigrina* (Eriksson, 1996). More relevant to the current study are observations reported by Bowman *et al.* (1995) that NOS activity in whole *P. redivivus* extract was partially dependent on exogenous calmodulin and completely dependent on Ca^{2+}.

To investigate the possible role of endogenous co-factors, purification of tissue extract was employed using ADP-Sepharose. Mammalian NOSs have been purified using a variety of chromatographic protocols but by far the most effective involve the use of 2′,5′-ADP coupled to an insoluble matrix such as agarose or Sepharose. NADPH-requiring enzymes such as NOS are retained by 2′,5′-ADP agarose and can then be eluted from this substrate by displacement with excess NADPH (Bredt & Snyder, 1990). We have applied this technique to purify NOS from extracts of *A. suum* neuromuscular tissue. However, unlike rat cerebellum, purification using 2′,5′ ADP-Sepharose resulted in an almost complete loss of *A. suum* NOS activity, thus co-factor dependence studies on purified enzyme could not be performed. The loss in NOS activity may be due to a failure to bind to the ADP-Sepharose, indicating that the enzyme is not NADPH dependent. Alternatively, selective binding of the *A. suum* NOS to ADP-Sepharose may be accompanied by loss of an essential co-factor which is not replaced in the assay system used. Another explanation is that the loss of NOS during the purification processing reduces the enzyme activity to a level below the limit of detection for the assay. Loss of rat neuronal NOS activity was also observed, when crude extracts of rat cerebellum were purified using the same procedure. However, since the starting rat crude extract possessed a very high activity, a loss of 80 % (as was the case in some experiments) did not eliminate detectable rat NOS activity altogether. Thus, the inability to purify *A. suum* NOS does not exclude the possible presence of a NOS-like NADPH-dependent enzyme. For future investigations, a possible alternative approach for the rapid separation of *A. suum* NOS-like activity from unbound low molecular weight co-factors and substrate is the Centricon filtration method used in this study.

In another approach to assessing the co-factor dependency of the *A. suum* NOS activity, the effect of the Ca^{2+} chelating agents EDTA and EGTA at varying concentrations was determined. At a concentration of 1 mM EGTA/EDTA, citrulline production was partially reduced to an extent which would account for the reduced activity of *A. suum* NOS in co-factor-deficient incubations. However, addition of high concentrations of EDTA (25 mM) and EGTA (12·5 mM) dramatically increased citrulline production by *A. suum* NOS. A number of possible explanations can be suggested for the surprising results obtained with the higher concentrations of EDTA/EGTA used on *A. suum* preparations. At these high concentrations the chelating agents either removed a transition metal(s) that inhibits NOS, or removed a metal that is required for the activity of a molecule that inhibits NOS. Evidence exists suggesting an inhibitory effect of transition metals on rat NOS activity. Persechini, McMillan & Masters (1995) reported that neuronal NOS activity was completely and reversibly inhibited by Zn^{2+}. Zn^{2+} blocks NADPH-dependent reduction of heme iron in nNOS and also blocks the calmodulin-dependent superoxide-mediated cytochrome c reductase activity exhibited by nNOS. Mittal, Harrell & Mehta (1995) have shown the interaction of heavy metals with rat brain constitutive NOS. The cations tested; Zn, Hg, Ni, Mn, Cd, Pb and Ca, inhibited NOS activity. Calcium ions required pre-incubation with the enzyme preparation in order to inhibit NOS. The authors suggested that while Ca^{2+} modulates cNOS activity at regulatory sites, an inhibitory influence of metal cations may be exerted on the catalytic site(s) either by direct binding or by interfering with electron

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transfer during catalysis. Similarly, Muller (1994) using the invertebrate tissue from *Apis* and *Drosophila*, showed that Ca^{2+} concentration required for half maximal stimulation of NOS is 0·3 μM, and that if Ca concentration exceeds 0·3 mM the activity declines by 30–40 %. In the present study, *A. suum* NOS activity was examined using assay buffer containing 1 mM Ca^{2+}. The alternative explanation for our observation, was the removal by EGTA/EDTA of a cation acting as a co-factor for a NOS inhibitory molecule. The existence of an endogenous NOS inhibitor is a possibility, based on findings observed by Jaffrey & Snyder (1996). They have identified a 10 kDa rat protein that physically interacts with, and inhibits, the activity of nNOS. They designated the inhibitor PIN (Protein Inhibitor of nNOS). The rat protein showed 92 % amino acid identity with that of the nematode *C. elegans*. To date, there have been no reports of the biochemical characterization, including any cofactor requirements of this inhibitory protein. Its discovery in *C. elegans*, suggests that it may be present in *A. suum* tissue and it would be interesting to know its biological role in nematodes.

Other potential candidates as NOS inhibitors are the porphyrins, which require specific metals for their activity. Wolff *et al*. (1996) have shown that various NOS isoforms can be inhibited by porphyrins, e.g. protoporphyrin IX and the metalloporphyrin zinc protoporphyrin IX. Various components of the protoporphyrin pathway have been detected in *A. suum* tissue and perienteric fluid (see Barrett, 1981), and may exist in our tissue extract, thus potentially affecting *A. suum* NOS activity.

The addition of NO donors e.g. sodium nitrite and hydroxylamine increased basal tone of *A. suum* dorsal muscle strip and reduced the size of muscle contractions induced by the excitatory neurotransmitter ACh. High concentrations of these weak donors were required. The biological effectiveness of nitric oxide donors depends on their NO-generating potency and their ability to cross cell membranes (Feelisch & Noack, 1987; Feelisch, 1991). Sodium nitrite is a weak NO releaser under physiological conditions. NO formation results from the decomposition of free nitrous acid and is pH dependent (acidic conditions) (Feelisch, 1991). Hydroxylamine is metabolized to NO by intracellular enzymes such as catalase (Murad, 1994), which may explain the high concentrations of hydroxylamine required to modulate the ACh responses of *A. suum* muscle strips (see Murad, 1994). In addition, the NO generated by these NO donors may be scavenged by the haemoglobin in the lateral lines of the muscle strip preparation, thus reducing the amount of free NO available to act on muscle cells. Recently, Minning *et al*. (1999) showed that *A. suum* haemoglobin functions as a deoxygenase, using NO to detoxify oxygen. Taken together, these factors may explain why our data are variable and the requirement for very high concentration of the NO donors to affect both basal muscle tension and the contractions induced by ACh.

The preliminary data obtained with these weak NO donors suggest the possibility that NO may be utilized as a signalling molecule in *A. suum* neuromuscular system. In mammalian systems, the major target for NO is the guanylate cyclase/cGMP pathway. The various components of the guanylate cyclase/cGMP system have been identified in nematodes. Guanylate cyclase and cGMP have been identified and determined in extracts of the free-living nematode, *P. redivivus* (see Willet, 1980), while Thalhofer & Hofer (1989) have purified cyclic GMP-dependent protein kinase from *A. suum*. The activation of the cGMP pathway in mammalian smooth muscles is generally associated with relaxation of the tissue. However, NO donors in the present study elicited an increase in basal tension, which cannot be explained by the traditional mechanism proposed for NO as an intracellular signal. Recently, evidence has emerged suggesting additional and alternative pathways as the mode of action of NO. Of interest, is the role of NO in mobilizing intracellular levels of Ca^{2+} from microsomal stores, which may occur via 1 of 3 mechanisms. (1) cGMP and the proposed endogenous ligand of ryanodine receptors, cADPR (Galione *et al*. 1993; Willmott *et al*. 1996; Berridge, 1993). (2) A direct action of NO on ryanodine receptors (Stoyanovsky *et al*. 1997). (3) The interaction of NO with reactive oxygen intermediates which have been reported to modulate Ca^{2+} flux at the sarcoplasmic reticulum by redox-sensitive thiol targets on Ca^{2+}-release channels, thus promoting Ca^{2+} release (Kobzik *et al*. 1994). The increase in intracellular Ca^{2+} by either method, would result in an increase in force of contraction, thus possibly explaining the increase in basal tension observed in the present study upon addition of NO donors to the dorsal muscle strip.

The inhibitory effects of NO donors on the ACh elicited contractions may be explained by the classical pathway proposed, involving the formation of cGMP and induction of muscle relaxation (Clementi & Meldolesi, 1997). An alternate mode of action of NO has been proposed in mammalian systems, which involves direct activation of K-channels in smooth muscle (Bolotina *et al*. 1994). This would act as a shunt from the depolarization induced via the activation of nicotinic receptors on *A. suum* muscle and reduce the effect of ACh on force of contraction. The possibility that NO interferes with ACh *via* this mechanism, would require electrophysiological examination.

This study is not the first to functionally investigate the potential role of the NO system in *A. suum*. Bowman *et al*. (1995) examined the effects of the NO donor sodium nitroprusside and NOS

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inhibitors on *A. suum* muscle strips. Unlike the present study, they observed a reduction in muscle tone, i.e. relaxation upon addition of NO donor. These differences may be explained by the model systems used. In the current study, the direct effects of NO donors on resting muscle tension were examined, producing an increase in muscle tone, while Bowman *et al.* (1995) examined the effect of NO donor on pre-contracted muscle strip (by pre-treating with levamisole) and observed a relaxation of the tissue.

In summary, the data presented in this study reveal that the neuromuscular system of *A. suum* contains a constitutive NOS-like enzyme, which catalyses the formation of NO and citrulline from L-arginine. The enzyme activity is only partially dependent on the classical mammalian NOS co-factors. Evidence using EGTA/EDTA suggested a partial dependence on Ca^{2+} for activity. This could indicate the presence of a different NOS isoform in parasitic nematodes, or the presence of more than one isoenzyme. The low enzyme activity in comparison to rat cerebellar NOS, may be due to a less pure preparation of neuronal tissue from *A. suum* nerve cord, coupled with the presence of non-optimum reaction conditions. Evidence is also presented for a role for NO as a neuromodulator suggesting its use in intercellular signalling. Further functional and biochemical assays are required to investigate the role of NO in cell signalling in *A. suum* and to further characterize this isoform of NOS. Molecular cloning of *A. suum* NOS-like enzyme would considerably strengthen the notion that the biochemical and histochemical data reflect, at least in part, the presence of NOS. The possible existence of a biologically active NOS isoenzyme in parasitic nematodes, that differs from mammalian NOS, offers a promising potential alternative site for drug targeting for future generations of anthelmintics.

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