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Pharmacological properties of non-adrenergic, non-cholinergic inhibitory transmission in chicken gizzard

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1 Non-adrenergic, non-cholinergic (NANC) inhibitory transmission in chicken gizzard was studied by use of intracellular microelectrode techniques. Changes in membrane potential in response to NANC nerve stimulation were recorded in the gizzard smooth muscle pretreated with atropine (1 μM) and guanethidine (1 μM).

2 Field stimulation of the intramural nerves (FS) evoked inhibitory junction potentials (i.j.ps) which were abolished by tetrodotoxin (1 μM), but not inhibited at all by K^+ channel blockers including apamin (0.5 μM), tetraethylammonium (TEA, 10 mM), charybdotoxin (0.2 μM) and glibenclamide (10 μM).

3 N^G-nitro-L-arginine (300 μM), an inhibitor of nitric oxide (NO) synthase, inhibited i.j.ps. The effect was reversed by L-arginine (3 mM), but not by D-arginine (3 mM).

4 8-Bromo cyclic GMP (100 μM), a membrane permeable analogue of cyclic GMP, produced a membrane hyperpolarization which was blocked by TEA (10 mM) or glibenclamide (10 μM).

5 NO at concentrations of up to 400 μM affected neither i.j.ps nor resting membrane potential. On the other hand, NO (80 μM) caused the membrane to hyperpolarize in the smooth muscle of guinea-pig ileum.

6 These results suggest that in the chicken gizzard, NANC i.j.ps may not arise from opening of conventional types of K^+ channel and that NO seems unlikely to be involved in the generation of i.j.ps. A possible mechanism by which the inhibitory effect of N^G-nitro-L-arginine on i.j.ps was brought about will be discussed.

Keywords: Non-adrenergic non-cholinergic innervation; inhibitory junction potential; apamin; nitric oxide; potassium channel blockers; smooth muscle; chicken gizzard

Introduction

The gizzard, or muscular stomach, is the most conspicuous organ of the digestive tract of the bird and receives extrinsic fibres from the vagus and the sympathetic system (Nolf, 1934 a, b). The organ has its own intramural ganglia forming Auerbach's plexus. Electrophysiological studies have revealed a non-adrenergic, non-cholinergic (NANC) inhibitory innervation of postganglionic fibres to gizzard smooth muscle (Bennett, 1969 a, b; Ohashi *et al*., 1993). The NANC inhibitory junction potentials (i.j.ps) can be evoked by electrical stimulation of the vagus nerve trunk or of the intramural nerves after blockade of both cholinergic and adrenergic transmission. The electrophysiological properties of the inhibitory transmitter are comparable to those of the NANC inhibitory transmitter in the gastrointestinal tract of mammals (e.g. see Gillespie, 1962; Bennett, 1966; Bülbring & Tomita, 1967).

The NANC i.j.p. in the mammalian gastrointestinal tract consists of two distinct components; one is blocked by apamin (a polypeptide constituent of bee venom); the other resistant to the polypeptide (Niel *et al*., 1983; Bywater & Taylor, 1986). The predominance of the two components varies from one species to another and in different regions of the gastrointestinal tract of the same species (e.g. see Shuba & Vladimirova, 1980; Bauer & Kuriyama, 1982; Komori & Suzuki, 1986; Christinck *et al*., 1991; Kitamura *et al*., 1993; Bridgewater *et al*., 1995). Generally, as apamin is a selective blocker of the small conductance Ca^{2+}-activated K^+ channel (Blatz & Magleby, 1986), the apamin-sensitive i.j.p. is believed to arise from the opening of this type of K^+ channel. On the other hand, ionic channels which underlie the apamin-resistant i.j.p. are still elusive (Niel *et al*., 1983; Christinck *et al*., 1991; Crist *et al*., 1991a, b).

In the gastrointestinal tract of mammals, NO has been proposed as a neurotransmitter which mediates NANC i.j.ps (Stark *et al*., 1991; Thornbury *et al*., 1991; Cristinck *et al*., 1991; Ward *et al*., 1992b; Kitamura *et al*., 1993; He & Goyal, 1993). The NO hypothesis is based primarily on the observations that transmission is inhibited by NO synthase (NOS) inhibitors and the inhibitory effect is reversed by L-arginine which functions as a substrate for NO biosynthesis by NOS. In canine colon and rat gastric fundus, guanosine 3′:5′-cyclic monophosphate (cyclic GMP) has been propsoed to serve as a second messenger linking the NO signal to the change in membrane potential (Ward *et al*., 1992a; Kitamura *et al*., 1993). On the other hand, evidence against the NO hypothesis (Bridgewater *et al*., 1995), which suggests a possible role for adenosine 5′-triphosphate (ATP) (Burnstock *et al*., 1970), vasoactive intestinal peptide (Furness *et al*., 1981) and pituitary adenylyl cyclase activating peptide (McConalogue *et al*., 1994) as a neurotransmitter generating i.j.ps also exists.

The present study was designed to clarify properties of NANC i.j.ps recorded intracellularly in avian gizzard, in an attempt to assess a possible involvement of NO in their generation.

Methods

Young male chicks, aged 10–60 days (Rhodhorn, GOTO 360), were stunned and killed by exanguination. The gizzard was removed and placed in a Petri dish filled with Tyrode solution (composition (mM): NaCl 136.7, KCl 2.7, NaH_2PO_4 0.4, $NaHCO_3$ 11.9, $MgCl_2$ 1.8, $CaCl_2$ 1.8, and glucose 5.6). A muscle strip (5–8 mm wide, 10–15 mm long) was dissected from the superficial muscle layer of the proximal intermediate muscle (Bennett, 1969a), placed, serosal side up, and pinned on the bottom of silicone layer of another Petri dish filled with Tyrode solution. Unwanted parts of the strip were trimmed away to give a final muscle preparation (3–6 mm wide, 8–13 mm long).

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The muscle preparation was pinned, serosal side up, to a small silicone rubber block. The rubber block and the preparation were immersed in an organ bath (1.5 ml) irrigated (4–5 ml min^{-1}) with Tyrode solution at 30°C and aerated with air in its reservoir (see below). At higher temperatures, difficulty in obtaining stable measurements of membrane potential response was found (Ohashi *et al.*, 1993).

Glass microelectrodes filled with 3 M KCl solution, with a resistance of 40–80 MΩ, were used to record, intracellularly, changes in membrane potential. A pair of silver wire electrodes (1 mm in diameter), one insulated with Araldite except for the tip and placed on the tissue, the other uninsulated and placed in the organ bath, were used for electrical field stimulation of the intramural nerves of the muscle preparation. Nerve stimulation was achieved with square wave pulses (0.3 ms, 50 V) delivered by a stimulator (Nihon Kohden, MSR-3R; Japan). To record membrane potential responses to intramural nerve stimulation, a microelectrode was inserted into a smooth muscle cell located within 2 mm from the stimulating electrode. An experiment, in which the effect of one or more substances on membrane potential was studied, was performed in the same cell; if the microelectrode was dislodged in the course of the experiment, another adjacent cell was impaled. Membrane potential changes were displayed on a cathode-ray oscilloscope (Nihon Kohden, VC-11; Japan) and recorded with a film-moving camera (Nihon Kohden, RLG-6101; Japan).

Muscle preparations from guinea-pig ileum were prepared as follows: a segment of the ileum (3–5 cm long) was removed from male guinea-pigs (350–400 g) stunned and then killed by exanguination. The intestinal segment was dissected longitudinally and rinsed in Tyrode solution, from which a muscle strip (approximately 10 mm × 10 mm) was prepared. The muscle preparation was pinned serosal side up on the rubber block and perfused with Tyrode solution as described for the gizzard muscle preparation.

Muscle preparations were equilibrated with Tyrode solution for 45–60 min and then atropine (1 μM) and guanethidine (1 μM) were given to block cholinergic and adrenergic transmission, respectively. They continued to be present throughout the experiment, unless otherwise stated.

Drugs used were atropine sulphate, L-arginine, D-arginine, tetraethylammonium chloride (TEA) (from Wako Pure Chem., Tokyo, Japan), guanethidine sulphate (from Tokyo Kasei, Tokyo, Japan), tetrodotoxin (TTX), apamin, glibenclamide, 8-bromoguanosine 3′, 5′-cyclic monophosphate (8-bromo cyclic GMP), 8-bromoadenosine 3′, 5′-cyclic monophosphate (8-bromo cyclic AMP) (from Sigma Chem., St Louis, U.S.A.), charybdotoxin (from Peptide Institute, Osaka, Japan), verapamil chloride (from Nakarai Chem., Kyoto, Japan) and N^G-nitro-L-arginine (from Aldrich, Milwaukee, U.S.A.). The concentrations refer to those of the base in the bathing solution.

Stock solutions of drugs were dissolved in distilled water, made up at 100 or more times higher concentrations than those used for experiments and stored at either 4 or −20°C. A stock solution of nitric oxide (NO) was prepared on the day of its use in the following way: 2 ml volumes of NO gas were removed by a syringe from a tube connected to a NO gas cylinder (from Daido, Gifu, Japan) and injected into 100 ml Tyrode solution which was deoxygenated by gassing with argon for 1.5–2 h and packed in a tightly-sealed container. A maximal possible concentration of NO in the stock solution was calculated to be 820 μM at room temperature. NO stock solution was stored at 4°C for 2–5 h until use.

Application of any drug to a muscle preparation was performed by switching the perfusion solution from one reservoir of Tyrode solution to another reservoir of Tyrode solution to which the drug had been added to give the desired concentration. In experiments, in which NO was used, the perfusion solution was not aerated to minimize oxidation of NO. The time lag for drugs to reach the organ bath was 25–30 s.

All data are expressed as means ± s.e.mean, where n refers to the number of cells. Data were tested for statistical significance by Student's paired or unpaired t tests. Values of $P<0.05$ were considered significant.

Results

The mean resting membrane potential in smooth muscle cells of the gizzard was −56.8 ± 0.3 mV ($n=163$ in 56 preparations). In 10% of preparations, a small spontaneous membrane depolarization (2–5 mV) lasting 2–3 s occurred intermittently with an irregular rhythm.

Field stimulation of the intramural nerve fibres (FS) with single pulses elicited i.j.ps with a delay of 100–150 ms, as previously described (Ohashi *et al.*, 1993). The total duration of an i.j.p. was some 2 s, and the peak amplitude rarely exceeded 2 mV. FS with trains of pulses at frequencies of up to 0.5 Hz evoked discrete i.j.ps with constant amplitude (Figure 1Aa, b). Above 1 Hz, successive i.j.ps summed up to give a long-lasting hyperpolarization, as shown in Figure 1Ac and d. The hyperpolarization reached a peak 1–3 s after cessation of FS then returned gradually to the resting membrane potential, to be often followed by a rebound depolarization (Figure 1Ac and d). The peak amplitude of the hyperpolarizing response increased by increasing stimulus frequency or the number of pulses. With seven to ten pulses at 20 to 30 Hz, the amplitude reached some 10 mV. TTX (0.5 μM) abolished the responses (Figure 1B), indicating their origin in nerves, i.e. they are NANC nerve-mediated i.j.ps.

In the following experiments, except where indicated, FS with five pulses at 10 Hz was used.

Effects of K^+ channel blockers on i.j.ps

A variety of K^+ channel blockers were used at concentrations similar to or higher than those shown to be effective in visceral and vascular smooth muscles (see Bolton & Beech, 1993).

Apamin (0.5 μM), which blocks small conductance Ca^{2+}-activated K^+ channels, had no appreciable effect on i.j.ps (Figure 2a). The mean i.j.p. amplitude in six preparations was 6.0 ± 0.6 mV before and 6.5 ± 0.7 mV ($n=6$) 4–6 min after application of the K^+ channel blocker. The difference between

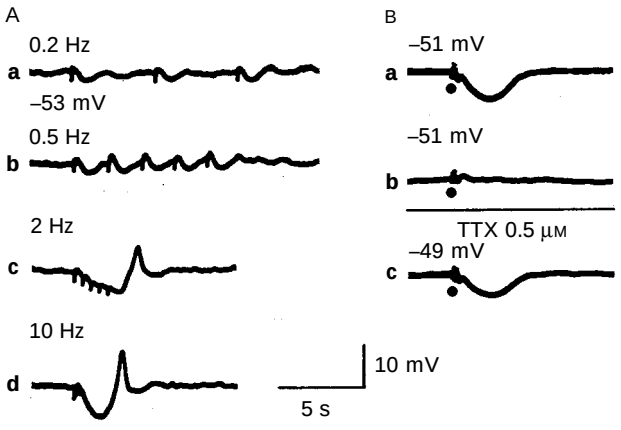

Figure 1 Non-adrenergic, non-cholinergic i.j.ps recorded intracellularly from smooth muscle cells of chicken gizzard. I.j.ps were evoked by field stimulation (FS) of the intramural nerves with square-wave pulses of 0.3 ms. (A) Responses to a train of three pulses at 0.2 Hz (a) and to a train of five pulses at 0.5 Hz (b), 2 Hz (c) and 10 Hz (d). (B) Responses to a train of five pulses at 10 Hz (●) before (a) and 3 min after application of 0.5 μM tetrodotoxin (TTX) (b) and 5 min after its removal (c). (A) and (B) are from different preparations. The resting membrane potential is indicated on the left hand side. Muscle preparations were perfused throughout with Tyrode solution containing 1 μM atropine and 1 μM guanethidine in this and the following figures, except where indicated.

the two mean values was not statistically significant (Figure 2e). Substantially similar results were obtained with TEA, a nonselective K^+ channel blocker, charybdotoxin, a blocker of the large conductance Ca^{2+}-activated K^+ channel or glibenclamide, a blocker of ATP-sensitive K^+ channel (Figure 2b, c and d). The mean amplitudes of i.j.ps before and after application of the individual drugs were 5.2 ± 0.3 and 5.6 ± 0.4 mV ($n=5$ in 5 preparations) for 10 mM TEA, 5.6 ± 0.6 and 5.2 ± 0.3 mV ($n=3$ in 3 preparations) for 0.2 μM charybdotoxin and 5.2 ± 0.4 and 5.3 ± 0.3 mV ($n=3$ in 3 preparations) for 10 μM glibenclamide, respectively (Figure 2e). A combination of 10 mM TEA with 0.5 μM apamin ($n=2$), 10 μM glibenclamide ($n=2$) or 0.2 μM charybdotoxin ($n=2$) did not have any noticeable effect on i.j.ps either. Apamin, TEA and charybdotoxin, but not glibenclamide, decreased the resting membrane potential by 3–8 mV.

The results suggest that the i.j.p. is not due to opening of the pharmacologically characterized types of K^+ channels.

Effect of N^G-nitro-L-arginine on i.j.ps

Effect of the NOS inhibitor, N^G-nitro-L-arginine (Moncada *et al.*, 1991) on i.j.ps was investigated in three different preparations. In each, i.j.ps were recorded from many cells before and during application of 300 μM N^G-nitro-L-arginine for 30 min (see Figure 3A), and i.j.ps recorded during the two different periods in the three different preparations were pooled separately. Further, i.j.ps recorded during the period of the drug application were divided into bins every 5 min. The mean amplitudes estimated for i.j.ps in every bin were compared with those for i.j.ps in the control period. As shown in Figure 3B, the amplitude of 4.9 ± 0.2 mV ($n=55$) was decreased by the NOS inhibitor. The maximal effect (1.7 ± 0.5 mV, $n=7$) was attained in 15–20 min after application of the drug, and sustained until its removal. The inhibitory effect was completely reversible so that the mean i.j.p. amplitude increased to 4.9 ± 0.5 mV ($n=21$), as high as the control, 40–60 min after its removal (Figure 3B).

The NOS inhibitor also decreased the resting membrane potential significantly from -56.0 ± 0.6 mV ($n=55$) to -51.2 ± 2.3 mV ($n=7$) ($P<0.05$). The depolarizing effect developed and disappeared after its removal with the same time course as that of its inhibitory effect on i.j.ps.

After the inhibitory effect of 300 μM N^Gnitro-L-arginine on i.j.ps had reached a maximum, either L- or D-arginine (3 mM) was applied and allowed to act for 20 min (see Figure 4A, B). Thereafter, i.j.ps were recorded from different cells over a period of 30 min. In the case of L-arginine, the mean i.j.p. amplitude was increased to 4.6 ± 0.4 mV ($n=17$) from 1.6 ± 0.3 mV ($n=16$) in the presence of N^G-nitro-L-arginine alone, that is, the i.j.p. amplitude returned to the level before application of the NOS inhibitor (4.5 ± 0.4 mV, $n=23$) (see the

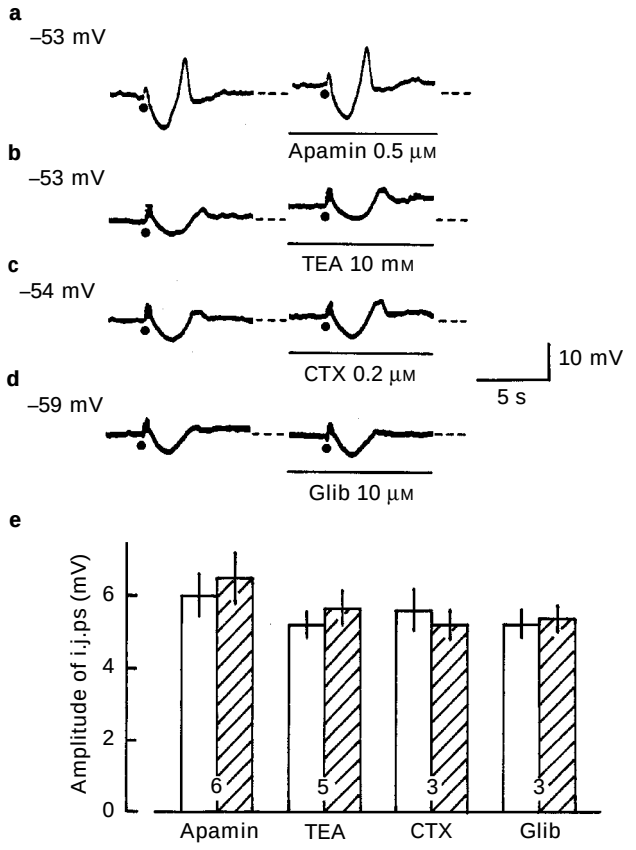

Figure 2 I.j.ps evoked by FS (five pulses at 10 Hz, ●) with supramaximal voltage before and after application of K^+ channel blockers. In (a)–(d), left hand side, control; right, after application of 0.5 μM apamin, 10 mM TEA, 0.2 μM charybtotoxin (CTX) or 10 μM glibenclamide (Glib), respectively, recorded from the same cell. The resting membrane potential before application of each K^+ channel blocker is indicated on the left, its level is also indicated by a dashed line. Traces (a)–(d) are from different preparations. (e) Comparison of i.j.p. amplitudes between before (open columns) and 4–6 min after application of apamin, TEA, CTX or Glib (hatched columns). Each column represents the mean of the indicated number of measurements. Vertical lines indicate s.e.mean. Note no significant effect of any K^+ channel blocker on the i.j.ps.

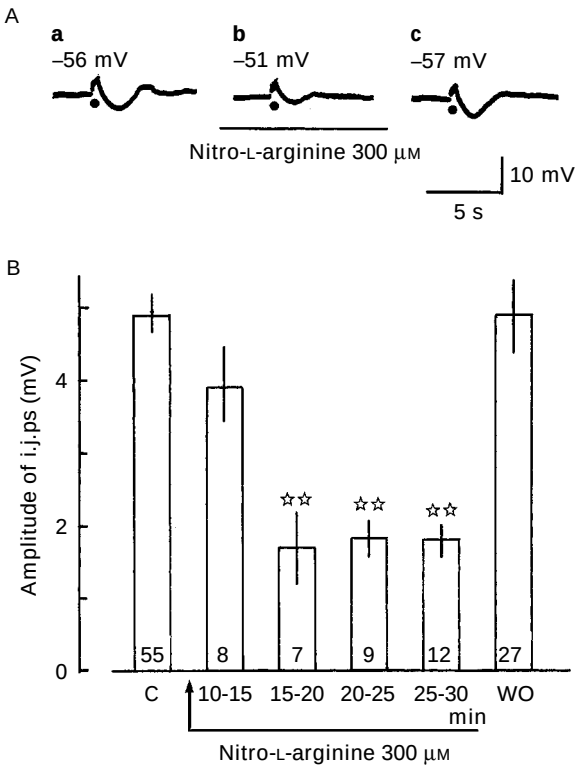

Figure 3 Effect of N^G-nitro-L-arginine on i.j.ps evoked by FS (five pulses at 10 Hz, ●) with supramaximal voltage. (A) I.j.ps with the resting membrane potentials before (a) and 20 min after application of N^G-nitro-L-arginine (300 μM) (b) and 30 min after its removal (c), respectively, in three different cells in the same preparation. (B) A summary of the effects of N^G-nitro-L-arginine on the FS-induced i.j.ps. Pooled data from three preparations were used to estimate mean amplitudes of the i.j.ps before (C) and during application of the drug and after its removal (WO). The data obtained in the presence of the drug were divided into bins every 5 min, by taking the beginning of its application as 0 min (see abscissa scale). Ordinate scale; i.j.p amplitude (mV). Each column represents the mean of the indicated number of measurements. Vertical lines indicate ± s.e.mean. ☆☆Statistically different ($P<0.01$) from the mean value for control (C). See text for details.

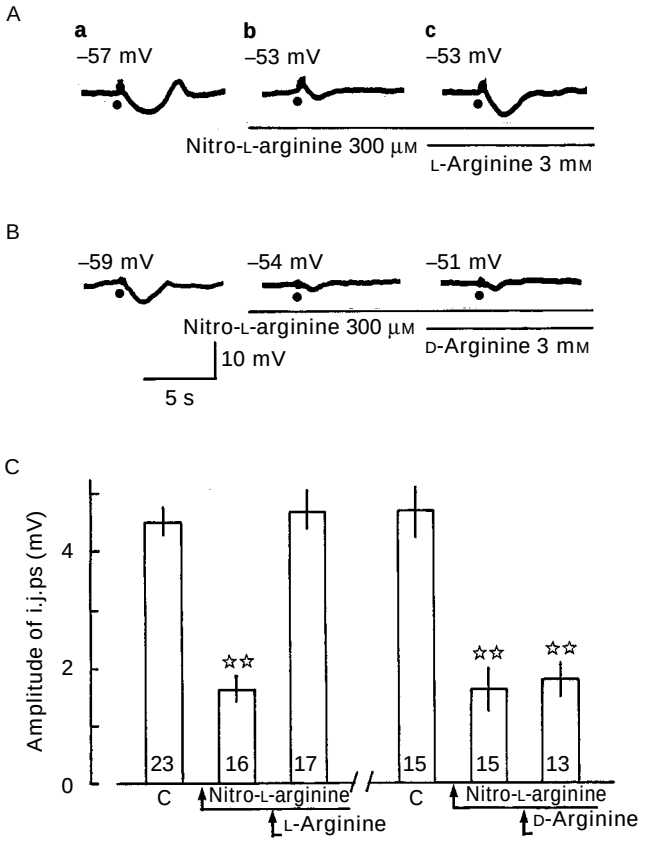

Figure 4 Effects of L- and D-arginine on the inhibition by N^G-nitro-L-arginine of i.j.ps evoked by FS (five pulses at 10 Hz) with supramaximal voltage. (A) and (B) FS-induced i.j.ps (●) before (a) and after application of 300 μM N^G-nitro-L-arginine (b) and after its combined application with 3 mM L-arginine or 3 mM D-arginine, respectively, (c). Trace (A) from three different cells in one preparation, and (B) from three different cells in another preparation. The resting membrane potentials of the cells are indicated. (C) A summary of the effects of L- and D-arginine. The i.j.ps were recorded before (C) and 15–30 min after application of N^G-nitro-L-arginine and 20–50 min after additional application of either L- or D-arginine (see abscissa scale). Ordinate scale; i.j.p. amplitude (mV). In each case of L- and D-arginine, pooled data from two preparations were used for measurements of i.j.p. amplitude. Each column represents the mean of the indicated number of measurements. Vertical lines indicate s.e.mean. ☆☆Statistically different ($P<0.01$) from the control (C). Note that L-arginine, but not D-arginine, reversed the effect of N^G-nitro-L-arginine.

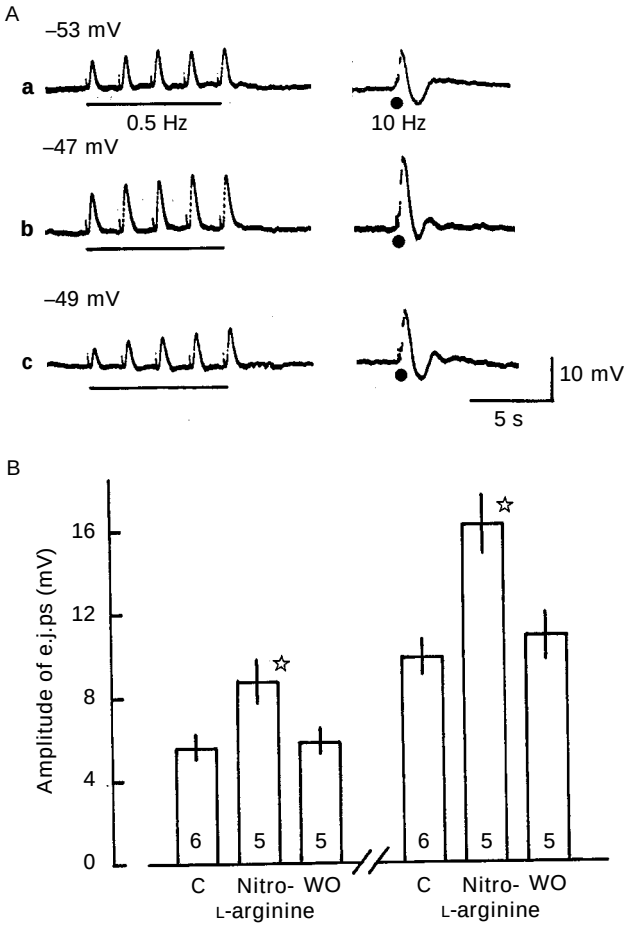

Figure 5 Effect of N^G-nitro-L-arginine on cholinergic e.j.ps recorded from smooth muscle cells of chicken gizzard. Muscle preparations were perfused with Tyrode solution containing 1 μM guanethidine and 2 μM verapamil, but without atropine. In (A), e.j.ps were evoked by FS with a train of five pulses at 0.5 Hz (left) and 10 Hz (right), as indicated by solid lines and closed circles, respectively. (a) Control; (b) 30 min after N^G-nitro-L-arginine (300 μM); (c) 40 min after its removal. Traces (a–c) are from three different cells in one preparation. (B) A summary of the effects of N^G-nitro-L-arginine on e.j.p. amplitude (the fifth e.j.p. of successive ones evoked with a train of five pulses at 0.5 Hz; the left set of three columns) and on the depolarizing component of biphasic responses to a train of five pulses at 10 Hz (the right set of three columns). The three columns in each set (left to right) represent control (C) and 15–30 min after N^G-nitro-L-arginine and 30–50 min after its removal (WO). Each column represents the mean of the indicated number of measurements in the preparation. Vertical lines indicate s.e.mean. ☆Statistically different ($P<0.05$) from the control (C).

left set of three columns in Figure 4C). The resting membrane potential was also increased to -56.0 ± 1.0 mV ($n=17$) from -53.5 ± 1.5 mV ($n=16$), but it was still significantly smaller ($P<0.05$) than the value before application of N^G-nitro-L-arginine (-59.0 ± 0.8 mV, $n=23$). D-Arginine was without effect on i.j.p. amplitude and the resting membrane potential. The mean i.j.p. amplitudes before and after D-arginine application were 1.6 ± 0.5 mV ($n=15$) and 1.7 ± 0.4 mV ($n=13$), respectively (see the right set of three columns in Figure 4C), and the resting membrane potentials were -53.5 ± 2.0 mV ($n=15$) and -51.5 ± 3.0 mV ($n=13$), respectively.

The results are consistent with the idea that NO is involved in the generation of NANC i.j.ps.

Effect of N^G-nitro-L-argining on cholinergic e.j.ps

In this series of experiments, atropine was omitted from the perfusion solution and verapamil (2 μM), a Ca^{2+} channel blocker, was used to prevent an action potential from discharging.

FS with a train of pulses at 0.5 Hz elicited discrete cholinergic e.j.ps with a gradually-increasing amplitude, and the maximum amplitude was attained after the fourth or fifth pulse, indicating facilitation of excitatory transmission. At the higher frequency of 10 Hz, a biphasic change in membrane potential occurred, which consisted of an initial depolarization resulting from summation of e.j.ps and a subsequent hyperpolarization (Figure 5Aa). When N^G-nitro-L-arginine (300 μM) was applied, the discrete and summed e.j.ps were increased in amplitude (Figure 5Ab). In one preparation, the mean amplitude of the fifth e.j.p. evoked with a train of five pulses at 0.5 Hz was 5.6 ± 0.6 mV ($n=6$) before and 8.7 ± 1.1 mV ($n=5$) 15–30 min after application of the drug. The difference between the values was statistically significant ($P<0.05$) (Figure 5B). However, the amplitude ratio of the fifth e.j.p. to the first e.j.p. of 1.44 ± 0.12 ($n=5$) remained almost unchanged after application of N^G-nitro-L-arginine (1.55 ± 0.14, $n=6$). Of the biphasic responses to a train of five pulses at 10 Hz, the amplitude of the depolarizing component was increased from 9.9 ± 0.9 mV ($n=6$) to 16.2 ± 1.5 mV

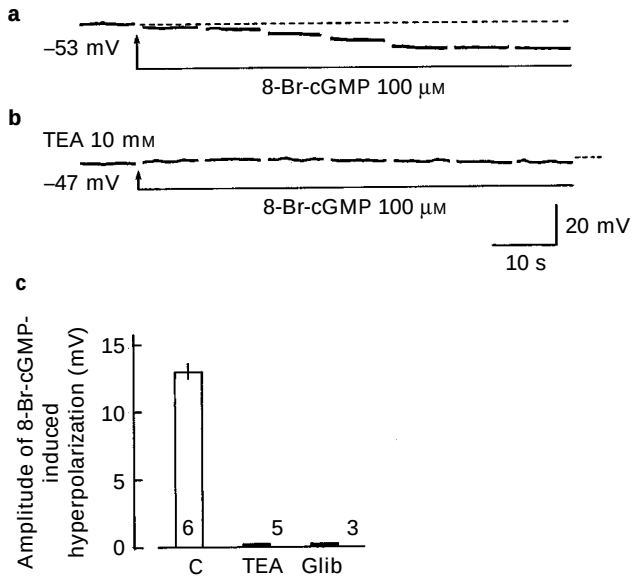

Figure 6 Effect of 8-bromo cyclic GMP (8-Br-cGMP) on the membrane potential. Traces (a) and (b) responses to 100 μM 8-Br-cyclic GMP in the absence and presence of 10 mM TEA, respectively. The recordings in (a) and (b) were taken from a continuous record; (left to right) immediately before and 1, 2, 3, 4, 5, 6 and 7 min after the beginning of the drug application. Time that elapsed between two successive traces was 52 s. The resting membrane potential before the drug application is indicated on the left and its level is also indicated by the dashed line. (c) Effects of 10 mM TEA and 10 μM glibenclamide (Glib) each on the 8-Br-cyclic GMP-induced hyperpolarization (C). Each column represents the mean of the indicated number of measurements in different preparations. Vertical line indicates s.e.mean. Note that TEA and Glib blocked 8-Br-cyclic GMP-induced hyperpolarizations, in contrast to their failure to block i.j.ps (see Figure 2).

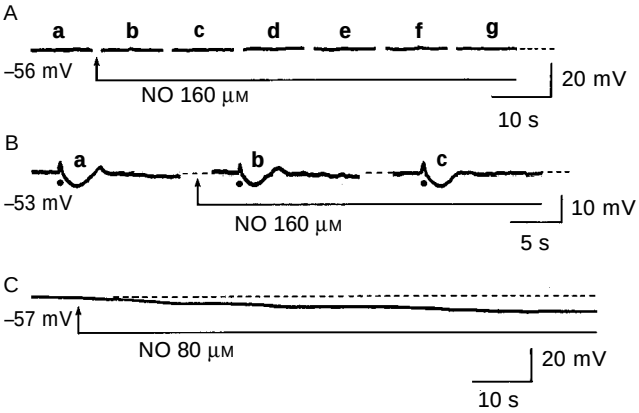

Figure 7 Effects of nitric oxide (NO) on the membrane potential and i.j.ps. (A) No appreciable change in membrane potential after application of 160 μM NO. The traces were taken from a continuous record; (a to g) immediately before and 0.5, 1, 2, 3, 4 and 5 min after the beginning of NO application. Times that elapsed between (b) and (c) and between two other successive traces were 20 s and 50 s, respectively. (B) I.j.ps evoked by FS (five pulses at 10 Hz, ●) before (a) and 3 min (b) and 5 min after application of 160 μM NO (c), in one cell. Dashed lines indicate the level of membrane potential before the NO application. (C) A hyperpolarizing response to 80 μM NO in the circular muscle preparation of guinea-pig ileum. The resting membrane potential before NO application in (A)–(C) is indicated on the left, its level is also indicated by the dashed line. See text for details.

($n=5$) with a statistically significant difference ($P<0.05$) (Figure 5B), whereas that of the hyperpolarizing component was decreased (see the right panels in Figure 5A). The action of N^G-nitro-L-arginine was reversible (Figure 5Ac and B). Similar results were obtained in three other preparations.

The potentiation of cholinergic e.j.ps appeared to reflect the action of N^G-nitro-L-arginine to suppress i.j.ps and therefore, the drug, at least at concentrations of up to 300 μM, is unlikely to exert a neurone-blocking action.

Effect of 8-bromo cyclic GMP on the membrane potential

Studies with canine colon and rat gastric fundus suggested that NO functions as a neurotransmitter generating i.j.ps and cyclic GMP serves as a second messenger linking the first signal of NO to the change in membrane potential (Ward *et al*., 1992a; Kitamura *et al*., 1993). In this respect, it was of interest to see whether cyclic GMP was involved in the generation of i.j.ps in chicken gizzard.

Figure 6a shows a hyperpolarization produced by 100 μM 8-bromo cyclic GMP, a membrane permeable analogue of the nucleotide. The hyperpolarization developed slowly to reach a maximum in 5–7 min after the beginning of its application and persisted until the drug was washed away. The mean maximum amplitude of hyperpolarizing responses in 6 preparations was 13.0 ± 0.8 mV ($n=6$). 8-Bromo cyclic AMP (100 μM), a membrane permeable analogue of cyclic AMP, produced no appreciable change in membrane potential in three preparations. In five muscle preparations pretreated with 10 mM TEA, 8-bromo cyclic GMP (100 μM) had little or no effect on the membrane potential, as shown in Figure 6b and c. A similar result was obtained with 10 μM glibenclamide ($n=3$ in three preparations; Figure 6c).

Taking the results together with the failure of TEA and glibenclamide to block the i.j.p. (see Figure 2), it seems unlikely that cyclic GMP is involved in the generation of i.j.ps in chicken gizzard.

Effect of NO on the membrane potential and i.j.ps

If NO serves as a neurotransmitter generating i.j.ps in chicken gizzard, exogenously-applied NO should be effective in hyperpolarizing the smooth muscle membrane. Experiments with NO were performed in air-free conditions (see Methods). It was confirmed that FS with a train of five pulses at 10 Hz elicited a similar hyperpolarization to that seen in normal conditions.

NO in solution at concentrations of 40 to 400 μM did not produce an appreciable change in membrane potential, as shown in Figure 7A ($n=11$ in nine preparations). The membrane potential measured 3–5 min after the beginning of application of NO (160 μM) was -56.1 ± 2.9 mV, which was very close to -56.3 ± 2.1 mV measured immediately before NO application ($n=6$ in six preparations). The application of NO did not significantly change the amplitude of i.j.ps either (Figure 7B). The mean amplitudes before and 3–5 min after application of NO (160 or 400 μM) were 5.1 ± 0.7 mV and 5.0 ± 0.7 mV ($n=5$ in four preparations), respectively.

In contrast, in muscle preparations from guinea-pig ileum, NO in solution (80 μM), when applied in the same way as for the gizzard muscle preparation, produced a prominent hyperpolarization in circular muscle cells of all the three preparations tested, as described by He and Goyal (1993). As shown in Figure 7C, the hyperpolarization increased gradually, reached a maximum of 14.1 ± 3.1 mV ($n=3$) in 1–1.5 min of the period of NO application, and then continued throughout the application period for 4–5 min.

Discussion

The present results showed that the NANC nerve-mediated i.j.p. in chicken gizzard is insensitive to apamin, a selective blocker of the small conductance Ca^{2+}-activated K^+ channel (Blatz & Magleby, 1986). The apamin concentration used,

0.5 μM, was higher than that (0.1 μM) required for inhibition of the i.j.ps in mammalian preparations including guinea-pig stomach and ileum (Komori & Suzuki, 1986; Nakao *et al*., 1986). Therefore, NANC nerves distributed to chicken gizzard seem unlikely to contain nerves which mediate apamin-sensitive effects, as demonstrated in opossum oesophagus (Jury *et al*., 1985) and chicken rectum (Komori & Ohashi, 1988).

In addition to apamin, charybdotoxin and glibenclamide, selective blockers of the large conductance Ca^{2+}-activated K^+ channels and ATP-sensitive K^+ channels, respectively, and TEA, a nonselective K^+ channel blocker, when each used at concentrations high enough to exert their effect in visceral and vascular smooth muscles (see Bolton & Beech, 1993), each failed to affect the i.j.ps in chicken gizzard. Given these observations, the i.j.ps may not be due to opening of pharmacologically characterized K^+ channels. The i.j.ps in chicken gizzard are very similar in both amplitude and time course to those in the rectum (Komori & Ohashi, 1988): the rectal i.j.ps are not associated with any consistent increase in membrane conductance and their amplitude changes independently of the electrical driving force for K^+. From these electrophysiological properties, it was concluded that an increase in membrane K^+ conductance is not involved in the generation of the i.j.ps. It can be assumed that ionic mechanisms other than activation of K^+ channels, such as closure of ion channels for which the equilibrium potential is more positive than the resting membrane potential, underlie NANC i.j.ps in avian gizzard and rectum.

The NANC inhibitory innervation to the taenia caeci, ileum and colon of the guinea-pig has been shown to be due to separate nerves, one of which mediates apamin-sensitive and the other apamin-resistant i.j.ps (Niel *et al*., 1983; Crist *et al*., 1991a; Zagorodnyuk & Maggi, 1994; Bridgewater *et al*., 1995). The apamin-resistant component is allegedly brought about by opening of pharmacologically unusual K^+ channels (Jury *et al*., 1985; Christinck *et al*., 1991), or closure of Cl^- channels (Crist *et al*., 1991a, b).

The inhibitory effect of N^G-nitro-L-arginine on i.j.ps in chicken gizzard is considered to reflect its action to inhibit NOS, since L-arginine, but not D-arginine, reversed the inhibitory effect. However, exogenous NO did not change the membrane potential and therefore the hyperpolarizing response to nerve stimulation cannot be explained by the idea that NO acts as a neurotransmitter generating i.j.ps, as suggested in the gastrointestinal tract of mammals (e.g. Stark *et al*., 1991; Christinck *et al*., 1991; Ward *et al*., 1992b; He & Goyal, 1993; Kitamura *et al*., 1993). It may be that NO in solution can mimic endogenous NO only when it is used in a more acceptable method, because of the labile nature of NO. On the other hand, NO in solution, when applied to the smooth muscle of guinea-pig ileum in the same way, increased the membrane potential. Failure of exogenous NO and NO-liberating compounds to cause membrane hyperpolarization has been described for some intestinal, tracheal and arterial smooth muscles of mammals. In the former two and the latter one, NO may be involved in NANC nerve-mediated and endothelium-dependent relaxation, respectively (Komori *et al*., 1988; Brayden, 1990; Suthamnatpong *et al*., 1994; Jing *et al*., 1995). The observations on the i.j.ps in chicken gizzard, together with data from previous studies, indicate that NO may not be important in the initiation of smooth muscle hyperpolarization, but it might have a predominant role either in stimulating or modulating the increase in membrane potential produced by an as yet unidentified NANC neurotransmitter. From this standpoint, the absence of effect of exogenous NO on the i.j.ps suggests that endogenous NO has fully exerted its action under such physiologically relevant conditions, rather than indicating its possible roles less likely.

In rat gastric fundus, N^G-nitro-L-arginine methyl ester did not reduce significantly NANC nerve-mediated relaxation unless it was used at concentrations 10–100 times higher than that required to block nerve stimulation-induced NO synthesis (Curro *et al*., 1996). In rat aorta, N^G-monomethyl L-arginine, another inhibitor of NOS, inhibited the relaxation produced by different compounds the chemical structure of which is not necessarily related to L-arginine, and the effect was thought to be produced through actions distinct from NOS inhibition (Thomas *et al*., 1989). The effect of these L-arginine derivatives, other than that of NOS inhibition, remains to be determined.

In canine colon and rat gastric fundus, NO, as a likely candidate for NANC neurotransmitters, has been suggested to activate the granulated isoform of guanylate cyclase to produce generation of cyclic GMP which plays a crucial role in cellular transduction mechanisms (Ward *et al*., 1992a; Kitamura *et al*., 1993). The suggestion is based on the observations that membrane hyperpolarization induced by NO and NO-liberating compounds and NANC nerve stimulation in the smooth muscle is resistant to methylene blue, an inhibitor of the soluble isoform of the enzyme (Ward *et al*., 1992a; Kitamura *et al*., 1993). In chicken gizzard, a membrane permeable analogue of cyclic GMP, 8-bromo cyclic GMP, was effective in causing smooth muscle membrane hyperpolarization and this hyperpolarizing effect was readily blocked by TEA and glibenclamide. Thus, the effect of 8-bromo cyclic GMP on membrane potential is inconsistent with its effect on the i.j.p., suggesting that cyclic GMP, and eventually NO, is not involved in the generation of the i.j.p. Now, the question arises as to how NO in solution had no effect on the membrane potential in chicken gizzard. It may be that the granulated isoform of guanylate cyclase is too small in amount to increase the cytosolic concentration of cyclic GMP to a level high enough to induce any change in membrane potential, since this form of the enzyme is known to vary widely in amount among different cell types (see Waldman & Murad, 1987).

In conclusion, NANC nerve-mediated i.j.ps in the chicken gizzard are independent of activation of pharmacologically characterized K^+ channels and seem likely to reflect the action of a compound which is distinct from NO, but remains as yet unidentified.

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