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The effect of inhibitors of nitric oxide biosynthesis and cyclic GMP formation on nerve-evoked relaxation of human cavernosal smooth muscle

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1 The inhibitory transmission in isolated preparations of cavernosal smooth muscle from human penis has been studied.

2 Electrical field stimulation (EFS; 2–64 pulses/train, 0.8 ms pulse duration, 10 Hz) evoked relaxation of preparations treated with guanethidine (50 μM). The EFS-evoked relaxations were atropine-resistant and tetrodotoxin-sensitive indicating their origin to be non-adrenergic, non-cholinergic (NANC) nerve stimulation.

3 EFS-evoked relaxation was attenuated dose-dependently by the nitric oxide (NO)-synthase inhibitor, L-N^G-nitro arginine (L-NOARG; 0.3–100 μM) but not by D-N^G-nitro arginine. The inhibitory effect of L-NOARG on transmission was antagonized by L-arginine (100 μM), a NO precursor, but not by D-arginine.

4 Incubation with methylene blue (10–50 μM), a known inhibitor of guanylate cyclase activation by NO, caused a concentration-related inhibition of EFS-evoked relaxation.

5 It is concluded that NANC nerve-evoked relaxation of human cavernosal smooth muscle is mediated by NO or a NO-like substance.

Keywords: Penis; penile erection; nitric oxide; non-adrenergic non-cholinergic transmission; methylene blue

Introduction

The erectile tissue of the human penis is contained within the corpora cavernosa and consists of endothelium-lined sinusoidal spaces surrounded by smooth muscle bundles. The relaxation of this cavernosal smooth muscle is a vital part of the sequence of psychological, neurological and vascular events necessary for erection to occur (Krane *et al.*, 1989). Previous studies on human isolated cavernosal tissue have shown that the relaxant response is nerve-mediated by a non-adrenergic, non-cholinergic (NANC) mechanism (Saenz de Tejada *et al.*, 1988b). Ambache *et al.* (1975) had isolated an inhibitory factor from a related smooth muscle, the bovine retractor penis (BRP), which closely mimicked the effects of stimulation of its NANC innervation. This inhibitory factor has since been identified as nitric oxide (NO, Martin *et al.*, 1988), the release of which mediates nerve-evoked relaxation in the BRP (Martin *et al.*, 1991). We have now investigated a possible role of NO in the nerve-evoked relaxation of human cavernosal smooth muscle by observing the effects of L-N^G-nitro arginine (L-NOARG), an inhibitor of NO biosynthesis (Moore *et al.*, 1990) and methylene blue, an antagonist of NO-mediated smooth muscle relaxation (Gruetter *et al.*, 1981). A preliminary account of some of the results described here has been presented at a meeting of The Physiological Society (Pickard *et al.*, 1991).

Methods

Ethical approval for the use of human tissue in this study was granted by the Newcastle Joint Ethics Committee.

Specimens of cavernosal tissue were obtained from 23 men undergoing penile surgery. At an appropriate stage during the operation a biopsy of cavernosal tissue was taken, placed immediately in chilled Krebs solution and transferred to the laboratory. The tissue was then either used straight away or stored overnight in fresh Krebs solution at 4°C. The tissue was prepared for the experiments by placing the biopsy on Krebs-soaked tissue paper in a Petri dish and cutting strips of cavernosal tissue measuring approximately 5 × 2 × 2 mm with a pair of fine scissors. The strips were then tied at each end with cotton threads and mounted in 1 or 2 ml organ baths containing Krebs solution maintained at 37°C and continuously aerated with 95% O_2 and 5% CO_2. The upper end of the strip was suspended from an isometric force transducer which was in turn connected to an amplifier, chart recorder and storage oscilloscope. An initial tension of 2 g was applied to the strip which was then allowed to equilibrate for 90 min. During this period the preparations were frequently washed with fresh Krebs solution (composition, mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2 and glucose 11).

Electrical field stimulation (EFS) was provided by a Grass S88 stimulator and applied via two parallel platinum wire electrodes set vertically within the organ bath at opposite sides of the suspended tissue. Parameters of EFS were as follows: trains of between 2 and 64 pulse at 100 s intervals, pulse duration = 0.8 ms, pulse-frequency = 10 Hz, supramaximal voltage (60 V). Once a stable resting tension had been reached the responses to EFS (2, 4, 8, 16, 32, 64 pulses/train) were recorded. Guanethidine (50 μM) was then added to the bath in order to block noradrenergic nerves and raise the smooth muscle tone. The responses to EFS were again recorded once the new level of tension had stabilised. If necessary, noradrenaline (NA; 10 μM) was also added to the bathing medium to maintain the level of tone required for the display of graded relaxant responses to EFS. In such cases the responses to EFS were recorded following its addition. The effects of treatment with atropine (3 μM) for a minimum of 20 min and tetrodotoxin (TTX; 0.3 μM for 5 min) on electrically-evoked relaxation were also tested on some strips.

Use of L-N^G-nitro arginine, L-arginine, D-N^G-nitro arginine and D-arginine

A train length that gave a consistent, sub-maximal relaxant response was chosen and repeated every 100 s. L-N^G-nitro

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arginine was then added to the bath in cumulatively rising concentrations of 0.3, 1, 3, 10, 30 and 100 μM. Each increment was added only after the response to EFS had been stable for 5 min. The effect of L-arginine on preparations treated with L-NOARG was tested in two ways. According to one procedure, after ascertaining maximum stable inhibition by L-NOARG (30 μM), L-arginine (100 μM) was added to the bath in the continued presence of L-NOARG and the exposure continued until the response to electrical stimulation had stabilized. In the second procedure, after obtaining maximum stable inhibition of the response to EFS by L-NOARG (30 μM), the drug was removed from the bath by repeated washes over a period of 10 min; when the relaxant responses to EFS had stabilized, L-arginine (100 μM) was introduced into the bath and the exposure maintained until the full effect of its introduction had been determined. The effect of substituting the optical isomers, D-arginine for L-arginine and D-N^G-nitro arginine (D-NOARG), was also tested on some preparations.

Use of methylene blue

Following the recording of control relaxant responses to EFS and glyceryl trinitrate (GTN; 0.3 μM), strips from 12 individuals were washed with fresh Krebs solution to remove guanethidine and NA and then incubated with methylene blue at concentrations of 10 μM and 50 μM for 1 h. At the end of this period, tone was re-established by adding guanethidine and NA without washing out methylene blue from the bathing solution. The responses to EFS and GTN (0.3 μM) were then again recorded. The effects of incubation with methylene blue (10 and 50 μM) on the magnitude of relaxation evoked by prostaglandin E_1 (PGE_1; 1 μM) were also tested on preparations from 3 individuals.

Statistical considerations

The amount of tissue obtained was insufficient to allow all experiments to be performed on isolated preparations from every individual. Results are expressed as mean ± s.e.mean of data from n individuals. Statistical significance was tested by Student's t test for paired data and accepted if $P < 0.05$ (one-tailed).

Drugs and solutions

Stock solutions of atropine sulphate (Sigma), L-arginine (Sigma), D-arginine (Sigma), glyceryl trinitrate (Lipha), guanethidine sulphate (CIBA), methylene blue (Sigma), L-N^G-nitro arginine (Sigma), prostaglandin E_1 (Upjohn), D-N^G-nitro arginine (Bachem), noradrenaline (Sigma) and tetrodotoxin (Sankyo) were all made up in distilled water and stored at −20°C.

Results

Response to electrical field stimulation

Prior to the addition of guanethidine, EFS produced varying degrees of contraction and relaxation in preparations from different individuals (Figure 1a,b,c). In contrast, EFS with guanethidine (50 μM) present in the bathing solution resulted in purely relaxant responses (Figure 1d). Preparations from 6 individuals required the addition of NA (10 μM) to ensure a sustained level of tone sufficient for recording consistent relaxant responses to EFS.

Once a stable tone had been achieved either with guanethidine (50 μM) alone or with the addition of noradrenaline (10 μM), a clear relationship emerged between the magnitude of the relaxant response and the number of pulses comprising the electrical stimulus (Figure 2). With trains of 16 pulses the relaxation began approximately 1 s after the start of the stimulus and reached its maximum at 8 ± 0.5 s ($n = 4$). Exposure of strips from 5 individuals to atropine (3 μM, 20 min) caused a slight, statistically insignificant, increase (3 ± 6%) in the degree of relaxation evoked by EFS. Tetrodotoxin (0.3 μM) completely abolished the relaxant response evoked by EFS in all strips tested ($n = 5$) within 5 min of its addition. There were no qualitative differences in the behaviour of strips between those used immediately and those stored for 24 h at 4°C. Tissue from 6 individuals was used both immediately and following 24 h storage. In these preparations, electrically evoked relaxation expressed as a percentage of maximal NA contraction increased by 20 ± 10% ($n = 6$, P = NS) in strips stored for 24 h compared to those used immediately.

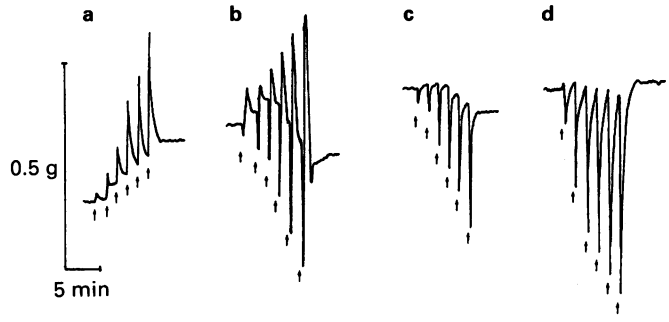

Figure 1 Isolated preparations of human cavernosal smooth muscle. Responses to electrical field stimulation (EFS) in preparations from 3 individuals in the absence of guanethidine (a–c) illustrating a purely contractile response (a), a mixed response (b) and a purely relaxant response (c). In the presence of guanethidine (50 μM) only relaxant responses were evoked by EFS and a typical example is shown in (d). In each panel, EFS comprised of trains of 2, 4, 8, 16, 32 and 64 pulses (10 Hz) in that sequence and the arrows mark the delivery of EFS.

Effect of L-N^G-nitro arginine

L-NOARG (0.3–100 μM), had no obvious effect on the tone of the preparation but inhibited electrically-evoked relaxations in a dose-dependent manner up to a maximum of 86 ± 4.5% at a concentration of 30 μM (Figures 3, 4). The inhibition of the relaxant response by L-NOARG was long lasting and only partially reversed by repeated washes with fresh Krebs solution (see below).

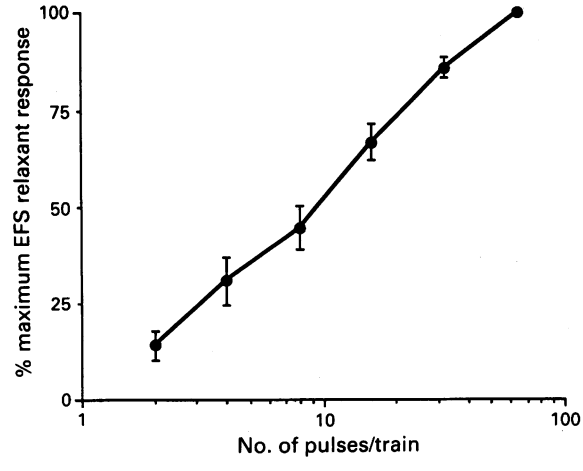

Figure 2 The effect of number of pulses comprising electrical field stimulation (EFS; 10 Hz) on the magnitude of the relaxant response. The responses are expressed as % of the maximal relaxant response. Each point is the mean response (bars showing s.e.mean) of 20 experiments conducted in the presence of guanethidine (50 μM).

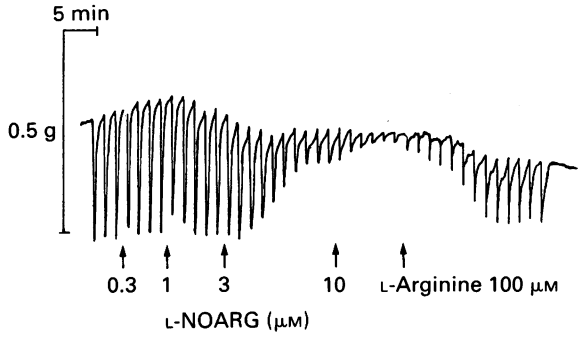

Figure 3 Isolated preparation of human cavernosal smooth muscle. A representative experiment demonstrating the inhibition of electrical field stimulation (16 pulses; 10 Hz)-evoked relaxation by cumulatively administered L-N^G-nitro arginine (L-NOARG, 0.3–10 μM) and the partial reversal of the inhibition by L-arginine (100 μM). Guanethidine (50 μM) was present throughout the experiment.

Identical parallel experiments with D-NOARG up to 100 μM on strips from 2 individuals, produced no inhibition of the relaxant response to EFS.

Effect of L-arginine

Resting tone and the relaxation produced by EFS were unaffected by the addition of L-arginine at a concentration of 100 μM. In the continued presence of L-NOARG (30 μM) the addition of L-arginine (100 μM) restored EFS-evoked relaxation to 26 ± 5.2% of the control ($n = 10$, $P < 0.01$); a representative tracing of an original recording is shown in Figure 3. Following the removal of L-NOARG from the bathing medium by repeated washes, there was partial recovery of the EFS-evoked relaxant response to 24 ± 6.6% of the control response ($n = 8$, $P < 0.01$), the recovery becoming stable between 10 and 25 min following washout. The subsequent addition of L-arginine further restored the response to 68 ± 7.7% of the control ($n = 8$, $P < 0.01$). This effect did not occur if D-arginine was substituted for L-arginine.

Effect of methylene blue

Following incubation with methylene blue (10 or 50 μM) for 1 h, tone was restored to 88 ± 7.6% of the original at a concentration of 10 μM ($n = 9$) and to 82 ± 4.5% at a concentration of 50 μM ($n = 12$) by the addition of guanethidine (50 μM)

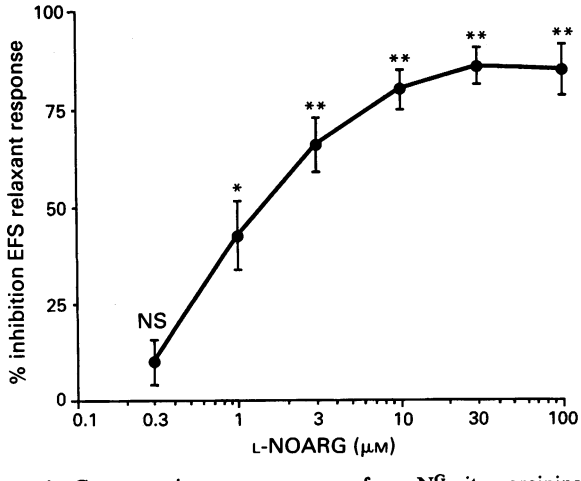

Figure 4 Concentration response curve for L-N^G-nitro arginine (L-NOARG) causing inhibition of electrical field stimulation-evoked (16 pulses; 10 Hz) relaxation of human cavernosal smooth muscle. Each point is the mean % inhibition with bars depicting s.e.mean ($n = 6$–14). Guanethidine (50 μM) was present throughout in each experiment. (NS = not significant; * $P < 0.01$; ** $P < 0.001$).

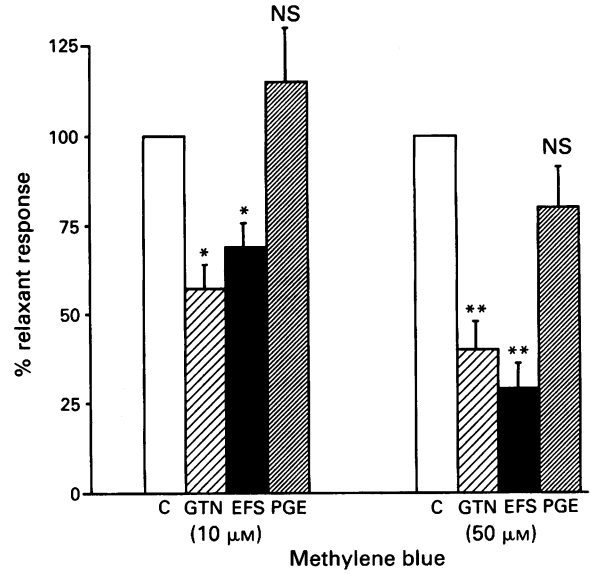

Figure 5 Effect of methylene blue 10 μM and 50 μM on the magnitude of relaxant responses of human cavernosal smooth muscle to electrical stimulation (EFS; 16 pulses; 10 Hz), glyceryl trinitrate (GTN; 0.3 μM) and prostaglandin E_1 (PGE, 1 μM). Each column represents the mean relaxant response following incubation with methylene blue expressed as a % of the original control response (C). The vertical bars indicate s.e.mean. NS = not significant; * $P < 0.01$; ** $P < 0.001$.

and NA (10 μM). The relaxation evoked by EFS was reduced by 31 ± 7% following incubation with methylene blue 10 μM ($n = 9$) and by 71 ± 7% with methylene blue 50 μM ($n = 12$, Figure 5). Methylene blue caused a similar reduction in magnitude of the relaxant response to GTN (0.3 μM), the respective reductions being 43 ± 6.8% ($n = 8$) and 60 ± 8.1% ($n = 10$, Figure 5).

The magnitude of relaxation produced by PGE_1 (1 μM) was unchanged following incubation with methylene blue at either concentration ($n = 3$, Figure 5).

Discussion

The relaxant responses of human cavernosal smooth muscle preparations to EFS in the present investigation were susceptible to full blockade by a low concentration (0.3 μM) of TTX, indicating that they originated from the stimulation of intrinsic nerves. The fact that these responses were evoked in the concurrent presence of a high concentration of an adrenergic neurone-blocker, guanethidine, should exclude the possibility of noradrenergic nerve stimulation in their genesis. It is also extremely unlikely that stimulation of cholinergic nerves by EFS and the resultant acetylcholine release contributes to any significant extent to the nerve-mediated relaxation of this tissue; treatment with atropine, although not routinely included in our experimental protocol, did not reduce the response to EFS. It is also relevant to note that in man, i.v. atropine fails to prevent penile erection evoked by tactile or visual stimuli (Adaikan *et al.*, 1986). Furthermore, to our knowledge clinical use of atropine-like agents is not associated with erectile failure. It seems reasonable therefore to conclude that the relaxant responses to EFS under the given conditions of our experimental protocol, originated as a result of the activation of NANC nerves supplying human corpus cavernosal smooth muscle. This conclusion is in agreement with similar conclusions drawn by others using human cavernosal smooth muscle preparations (Andersson *et al.*, 1983; Saenz de Tejada *et al.*, 1988b).

Of particular interest, in relation to the mechanism of NANC relaxation of human cavernosal smooth muscle are the findings of Martin *et al.* (1988, 1991). Bovine retractor

penis (BRP), a smooth muscle homologous to cavernosal smooth muscle, also contains a NANC inhibitory innervation (Klinge & Sjostrand, 1974); relaxation of BRP is an essential prerequisite for penile erection in this species (Kolliker, 1852) as is the relaxation of cavernosal smooth muscle in man (Saenz de Tejada et al., 1988a). Ambache et al. (1975) had isolated an inhibitory factor from BRP which closely mimicked the effects of activation of its inhibitory nerves. Martin et al. (1988) identified the active constituent of the inhibitory factor from BRP as NO and have very recently provided evidence showing that NO mediates the NANC nerve-evoked relaxation of BRP (Martin et al., 1991).

Two approaches have been adopted in the present investigation to examine the possibility that NO or a related substance serves as the mediator for NANC inhibitory transmission and the results, on both counts, are unequivocal. First, by using chemical agents which have a bearing on the physiological pathway for NO biosynthesis, evidence in favour of a mediator role of NO in inhibitory transmission has been accumulated. The amino acid L-arginine is the physiological precursor of NO in a chemical reaction catalysed by the enzyme NO synthase (Marletta et al., 1988). Several N^G-substituted analogues of L-arginine can competitively inhibit NO biosynthesis (Palmer et al., 1988), L-NOARG being a highly potent example (Ishii et al., 1990). In our experiments, L-NOARG (0.3–100 μM) caused a concentration-dependent inhibition of the relaxant response to EFS and the maximum inhibition in many experiments amounted to virtual extinction of the inhibitory neurotransmission. The NO precursor, L-arginine, on its own, had no effect on the inhibitory transmission but it is highly relevant that the inhibition of the transmission by L-NOARG was reversed by incubation with L-arginine both before and to a greater extent after the wash-out of L-NOARG, indicating the competitive nature of the antagonism between L-arginine and L-NOARG. The stereospecificity of both L-arginine and L-NOARG was demonstrated by the ineffectiveness of D-arginine and D-NOARG, adding further evidence that the L-arginine-NO pathway is the primary target for the action of L-NOARG. These results bear a striking resemblance to those from several other tissues in which inhibitory transmission has been postulated to be mediated by NO (Gillespie et al., 1989; Gibson et al., 1990; Tucker et al., 1990; Hobbs & Gibson, 1990).

The second approach for testing a possible role of NO or a related substance in the inhibitory transmission of human cavernosal smooth muscle has involved the determination of the effect of the oxidant, methylene blue on the relaxant response to EFS. It is now widely recognized that NO stimulates the activity of soluble guanylate cyclase and thereby causes a rise in guanosine 3':5'-cyclic monophosphate (cyclic GMP) levels in the smooth muscle leading to its relaxation (Kimura et al., 1975; Holzman, 1982). There is also evidence that stimulation by NO of guanylate cyclase activity and the consequent smooth muscle relaxation can be blocked by methylene blue (Gruetter et al., 1981; Martin et al., 1985). If the inhibitory transmission in human cavernosal smooth muscle is indeed mediated by NO, it should be possible to antagonize the inhibitory transmission by inhibiting guanylate cyclase. The use of methylene blue at 10 and 50 μM in the present investigation resulted in substantial reductions in the EFS-evoked relaxant responses of the cavernosal smooth muscle preparations. The degree of inhibition of the relaxant response to EFS by methylene blue was greater at 50 μM and, statistically, highly significant. The finding that methylene blue caused reduction in relaxation evoked by GTN strengthens the argument in favour of NO as the mediator of nerve-evoked relaxation in this tissue, since NO is known to be the active metabolite responsible for the smooth muscle relaxant action of GTN (Gruetter et al., 1981). The specificity of methylene blue as an inhibitor of the NO-cyclic GMP relaxant mechanism is also shown by its lack of effect on the relaxation produced by PGE_1, which has been shown to act via the generation of adenosine 3':5'-cyclic monophosphate (cyclic AMP) in a homologous smooth muscle preparation (Bowman & Drummond, 1984).

The results of the present investigation provide strong evidence supporting a role of NO or a NO-like substance in the nerve-mediated relaxation of cavernosal smooth muscle and therefore by implication in human penile erection. Three recent publications have considerable bearing on our conclusions regarding the role of NO in this tissue. The most recent of the three, by Holmquist et al. (1991) which appeared while the present manuscript was in preparation, found, in common with our findings, that L-NOARG inhibited NANC relaxation of human isolated corpus cavernosum and these authors have also concluded that NO is involved as a mediator in the relaxant response to EFS. In sharp contrast to our findings and those of Holmquist et al. (1991), Sjostrand et al. (1990) have reported a lack of blocking effect of L-N^G-monomethyl arginine (L-NMMA) on the NANC inhibitory transmission of human cavernosal tissue. L-NMMA, in common with L-NOARG, is a specific inhibitor of NO formation from L-arginine and therefore Sjostrand et al. (1990) interpreted their results to indicate that NO was not the mediator for the relaxation in response to EFS. A better understanding of the results of Sjostrand et al. (1990) becomes possible by examining the findings of Gillespie and his colleagues regarding the actions of L-NMMA and L-NOARG on NANC relaxation of the BRP (Gillespie & Xiaorong, 1989; Martin et al., 1991). They found that the NANC relaxation of BRP was blocked by L-NOARG but not by L-NMMA. Thus it would seem that the lack of susceptibility of NANC transmission to L-NMMA is shared by the two homologous tissues, human corpus cavernosum and BRP. It is probably due to some peculiarity of the NO synthase in the two tissues, and should not be interpreted as indicating an absence of involvement of the L-arginine-NO pathway in the NANC relaxant response. The involvement of this pathway in the inhibitory transmission of rabbit corpus cavernosal smooth muscle (Ignarro et al., 1990) reinforces the suspicion that it is likely to be widespread, transcending different mammalian species.

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