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Coexistence of non-adrenergic non-cholinergic inhibitory and excitatory neurotransmitters in a large neuronal subpopulation in the vaginal segment of the chicken oviduct

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Abstract

The presence, distribution and smooth muscle motor effects of galanin and pituitary adenylate cyclase activating peptide (PACAP) were studied in the nerves of the vaginal part of the oviduct of egg-laying hens. Galanin and PACAP immunoreactivity were found both in neuronal perikarya and nerve fibres within the wall of the vaginal segment. Both populations showed a similar distribution pattern. Particularly the circular muscle and the intramural vascular net were richly innervated. A few galanin- and PACAP-IR nerve fibres extended up to the mucosal folds. Multiple labelling showed galanin to be colocalised with PACAP as well as with vasoactive intestinal polypeptide (VIP) and nitric oxide synthase (NOS) in a large, partly intrinsic neuronal subpopulation innervating the smooth muscle wall. Pharmacological in vitro experiments showed that isolated vaginal muscle strips had a spontaneous basal activity that was not affected by the neuronal conductance blocker tetrodotoxin (TTX). Galanin induced concentration-dependent contractions that were TTX-insensitive. PACAP, VIP, nitric oxide (NO) and the NO donor nitroglycerin caused concentration-dependent relaxations that were TTX-insensitive. Electrical field stimulation of isolated muscle strips induced frequency-dependent relaxations that were blocked by TTX and reduced by the NOS blocker L-nitroarginine. These data provide evidence that the vaginal part of the oviduct contains a largely intrinsic, neuronal subpopulation, capable of releasing multiple non-adrenergic, non-cholinergic (NANC) motor agents for the control of local muscular activities. In addition, we provided pharmacological evidence that VIP, NO and PACAP exert an inhibitory and galanin an excitatory action on isolated muscle strips of the vaginal part of the chicken oviduct. Our results suggest that these NANC neurotransmitters play an important role in the regulation of neuromuscular activity in this region.

Keywords: Chicken; Lower oviduct; Oviposition; Motility; Autonomic nervous system

1. Introduction

The vaginal part of the chicken oviduct is a highly distensible muscular tube that allows the passage of the rigid shelled egg by a mechanism called oviposition. Functionally, when the egg is in the shell gland, the vagina is tonically closed to avoid premature oviposition. Subsequently, the strong contraction of the smooth musculature of the shell gland forces the egg through the vagina, which relaxes to accomplish oviposition. An alteration in normal avian distal segment tone and motility may lead to premature ovideposition (Lake and Gilbert, 1964), similarly to the abortion that can be observed in rat under experimental conditions (Doyle and Margolis, 1963). Oviposition occurs under neural control as well as under hormonal control, including the actions of arginine vasotocin (Rzasa and Ewy, 1970), which links specific receptors (Koike et al., 1988; Takahashi et al., 1992, 1998), and of ovarian prostaglandins (Wechsung and Houvenaghel, 1976). Sympathetic and parasympathetic nerves reach the distal region via the large blood vessels (Gilbert and Lake, 1963). Noradrenergic nerve

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fibres have been described in the smooth muscle layer (Bennett et al., 1973) and have been shown to be capable of inducing contraction of the oviductal wall (Verma et al., 1977; Crossley et al., 1980).

Recently, evidence has been obtained suggesting that motility of the vagina is controlled by non-adrenergic, non-cholinergic (NANC) transmitters. The vaginal wall has been reported to harbour a dense neuronal subpopulation containing vasoactive intestinal polypeptide (VIP) and nitric oxide (NO) that mainly innervates the thick muscle layer of this segment (Costagliola et al., 1997). VIP and NO are known to be inhibitory transmitters; an early study has shown VIP involvement in the relaxation of the vagina during oviposition (Houvenaghel and Wechsung, 1989). However, other NANC neurotransmitters, such as galanin and pituitary adenylate cyclase activating peptide (PACAP), may also be involved.

Galanin, a 29-amino acid residue neuropeptide that was first purified from the porcine small intestine (Tatemoto et al., 1983) has been reported in the oviductal musculature of quail, where it was found to evoke oviposition through a mechanism of induction of uterine and vaginal contractions (Li et al., 1996). This finding was further strengthened by the observation of galanin-binding sites in this muscle layer (Tsutsui et al., 1997). The sequence of the peptide has been well conserved among mammalian species; it is widespread in neural elements of distinct mammalian (Bauer et al., 1986; Barnes, 1987; Kaplan et al., 1988; Rokäeus and Carlquist, 1988; Hoyle and Burnstock, 1989, Lindh et al., 1989; Ahren et al., 1990) and avian organs (Luts et al., 1989; Norberg et al., 1991; Jozsa and Mess, 1993; Salvi et al., 1999). In mammals, galanin has been shown to play an important role as a neurotransmitter/neuromodulator substance in neuronal circuits controlling several functions (Ekblad et al., 1985; Stjernquist et al., 1988; Parsons et al., 1989; Bauer, 1990; Ulman et al., 1992). The mammalian and avian isoforms share the first 15 amino acid residues, but differ at several positions in the C-terminal part (Norberg et al., 1991). In addition, the C-terminal part is amidated in all known forms, including chicken (Norberg et al., 1991), but not in human (Schmidt et al., 1991). PACAP, a member of the VIP/secretin/glucagon family, was originally isolated from an extract of ovine hypothalamus on the basis of its ability to stimulate cAMP formation in rat pituitary cells (Miyata et al., 1989). PACAP is widely distributed in the brain and peripheral organs, such as the endocrine pancreas, gonads, respiratory and urogenital tracts, where it is involved in the regulation of important biological functions (for review see Vaudry et al., 2000). PACAP comprises 38 amino acid residues and is C-terminally α-amidated in mammals, but not in chickens (for review see Hoyle, 1998). The internal cleavage-amidation of the PACAP precursor can generate a 27-residue α-amidated polypeptide, PACAP-27. The latter is the biologically active region of PACAP. Its sequence shows 68% identity with VIP and shares with the latter common receptors with different affinities (PAC1, VPAC1, VPAC2; Harmar et al., 1998; Robberecht et al., 1998). The sequence of PACAP-27 has been preserved during evolution and exhibits in chickens only 1 amino acid substitution (Ser^2–Ile^2) compared to mammalian forms (for review, see Hoyle, 1998). PACAP has also been reported in the avian central nervous system (Peeters et al., 1998) and in gastrointestinal nerves (Sundler et al., 1992).

Several species-dependent colocalisation patterns of VIP, NO and PACAP have been disclosed in inhibitory motor neurons in peripheral organs, although more putative roles can be attributed. Galanin has been found in peptidergic nerves containing VIP and substance P (Zhu and Dey, 1992) and in adrenergic nerves (Ahren et al., 1990). Research on the motor activities of galanin in the gastrointestinal and genital smooth muscles of several mammalian species has yielded both stimulatory and inhibitory effects resulting from either a direct myogenic effect or an indirect action via the release of other neurotransmitters (Stjernquist et al., 1988; Botella et al., 1995).

Consequently, the present study was designed to (1) determine the distribution pattern of galanin and PACAP-27, their mutual relationships as well as their relation with VIP and NO, and with adrenergic nerves; and (2) to characterise pharmacologically the functions fulfilled by these neuronal messengers in the chicken vaginal segment.

2. Materials and methods

Fourteen 10–12-month-old egg-laying hens (layer breeder: reddish Isabrown) were fed with grain and water ad libitum. The animals were subdivided into two groups: animals used for immunohistochemical investigation at the light microscopic level ($n=6$) and animals used for pharmacological investigation of exogenously applied agonists and antagonists on vaginal smooth muscle contractility ($n=8$). All animals were anaesthetised by means of intravenous injection of an overdose of sodium pentobarbital. After laparotomy, the vaginal segment was dissected out and differently processed for either immunocytochemistry or pharmacological experiments (see below). All experimental procedures were approved by the local ethics committee of the University of Antwerp.

2.1. Immunohistochemistry

Tissue samples were fixed in Zamboni's solution (4% paraformaldehyde and saturated 0.2% picric acid in 0.1 M phosphate buffer, pH 7.4) for 2 h, then rinsed in phosphate-buffered saline (PBS) and transferred into 18% sucrose solution until further processing. Ten-micrometer-thick cryostat sections were mounted on chrome-alum-gelatin-coated glass slides, which were air-dried for 50 min at room temperature, washed in PBS and incubated in a blocking mixture containing 1% normal goat serum, 0.1% bovine

serum albumin and 0.1% Triton X-100 in PBS with 0.5% thimerosal, for 1 h at room temperature. (see Table 1 for details of the antisera used). Double labelling immunofluorescence was performed by incubating sections with a set of two primary antibodies raised in different host species for 16 h, followed by rinsing in PBS and visualised by secondary antisera conjugated to corresponding fluorochromes or biotin-coupled secondary antibodies, the latter revealed by streptavidin-linked fluorochromes. To obtain enhanced sensitivity and to allow combination of antisera raised in the same species, a biotin-conjugated TSA kit (Perkin Elmer Life Sciences NEL700) was applied according to the procedure for triple fluorescence staining with antibodies raised in the same species (Brouns et al., 2002, 2004). Briefly, after primary (overnight) incubation with the first rabbit polyclonal antibody (raised against PACAP, diluted 1:600,000), sections were consecutively incubated with biotin-conjugated Fab fragments of affinity-purified goat anti-rabbit immunoglobulins G (diluted 1:500, 1 h) and extravidin-horse radish peroxidase (diluted 1:1000 in PBS, 1 h). Between subsequent steps, sections were rinsed in PBS containing 0.05% Tween 20. They were further incubated with biotin-conjugated tyramide (diluted 1:100 in 'amplification solution') and staining was visualised by Cy3-conjugated streptavidin. The next step included the simultaneous incubation in the second primary antibody (a rabbit polyclonal one raised against galanin) and the third primary antibody (a mouse monoclonal one raised against VIP). These antibodies were visualised using an AMCA-conjugated donkey anti-rabbit IgG and an FITC-conjugated goat anti-mouse IgG, respectively.

All primary and secondary antibodies were diluted in PBS containing 10% normal goat serum, 0.01% bovine serum albumin (BSA), 0.05% thimerosal and 0.01% sodium azide. The specific immunoreaction was completely abolished by preabsorption of the diluted antisera with 20 μg/ml of the appropriate antigen (synthetic porcine galanin, synthetic porcine VIP, Peninsula, St-Helens, UK), omission of the primary antisera or replacement by an appropriate non-immune serum. Negative (staining) controls for the triple labelling were performed by omission of the primary or secondary antisera or substitution with non-immune serum as described by Brouns et al. (2002, 2004). After a final wash the slides were mounted in Vectashield (Vector, Burlingame, USA) and observed using an Olympus BX-50 fluorescence microscope equipped with a computerised stage and a CCD camera, which was coupled to a personal computer equipped with Analysis Pro 3.00 software (Soft Imaging System, Münster, Germany).

When the FITC fluorochrome was used, a punctate, unspecific, staining appeared in the mucosal epithelium of both positive and negative controls in all immunohistochemical stainings.

2.1.1. Quantification of the colocalisation of galanin immunoreactivity with vasoactive intestinal polypeptide immunoreactivity in vaginal nerve cell bodies

The proportion of neurons labelled for galanin that also colocalised VIP was estimated by cell counting on cryosections of the vaginal segment from two animals, double immunostained for galanin and VIP. The following proce-

Table 1

Primary antisera

Antigen	Host species	Working dilution	Supplier	Code
Galanin	rabbit, poly	1:3000 1:20,000	Biogenesis	4600–5004
nNOS	rabbit, poly	1:500	Gift from B. Mayer (Timmermans et al., 1994a,b)	
nNOS	mouse, mono	1:2000	Sigma	N2280
PACAP-27	rabbit, poly	1:8000 1:600,000[a]	Peninsula	IHC 8922
TH	mouse, mono	1:60	Boehringer Mannheim	BM1017381
VIP	mouse, mono	1:400	East Acres Biological	MaVIP

Secondary and tertiary antisera

Antigen	Host species	Conjugate	Work dilution	Supplier
Rabbit IgG	goat	FITC	1:100	Jackson Laboratories
Mouse IgG	goat	FITC	1:200	Jackson Laboratories
Rabbit IgG	donkey	AMCA	1:100	Jackson Laboratories
Rabbit IgG	goat	Biotinylated	1:500	DAKO
Mouse IgG	goat	Biotinylated	1:500	DAKO
		Cy3, streptavidin	1:9000	Jackson Laboratories
		FITC, streptavidin	1:1000	Jackson Laboratories

nNOS, neuronal (type I) nitric oxide synthase; PACAP-27, pituitary adenylate cyclase activating peptide-27; TH, tyrosine hydroxylase; VIP, vasoactive intestinal polypeptide.

FITC, fluorescein isothiocyanate; AMCA, 7-amino-4methylcoumarin-3-acetic acid; Cy3, Cy3™.

[a] Dilution used in the tyramide signal amplification method.

dure was used: starting from one defined point of the section and, moving across the slide in a systematic way, galanin-IR nerve cell bodies were identified. The proportions of neurons containing galanin (without VIP), VIP (without galanin) and both galanin and VIP were calculated. The counts of the cell bodies were performed using a 25× and 40× objective lens.

2.2. Pharmacology

2.2.1. Tissue preparation

After opening the abdominal cavity, the whole vaginal canal was removed while maintaining its cranio-caudal orientation, and immediately washed in Krebs-Ringer solution (118.3 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 0.026 mM CaEDTA and 11.1 mM glucose). After removal of the mucosa, four longitudinal and four circular muscle strips (length: ~ 7 mm, width: ~ 3 mm) were prepared from the upper-middle portions of the segment, mounted in organ baths (volume: 5 ml, filled with Krebs-Ringer solution, kept at 39 °C and aerated with 95% O_2/5% CO_2) and connected to a strain gauge transducer (UC2, Scaime, France) for continuous recording of isometric tension. Preliminary experiments earlier showed that a resting tension of ~ 0.5 and ~ 0.75 g resulted in optimal responses to noradrenaline (NA) in circular and longitudinal muscle strips, respectively. After the strips had been returned to their respective resting tensions, they were allowed to equilibrate at least 90 min before starting the experiments.

2.2.2. Experimental protocols

All experiments, except those with galanin, were performed in the presence of atropine (muscarinic receptor blocker, 10^{-6} M) and guanethidine (depleting neuronal adrenergic stores, 3×10^{-6} M) to block cholinergic and adrenergic neurotransmission. The effect of galanin was studied in control conditions (saline) and in the presence of propranolol and phentolamine.

To study inhibitory responses, muscle strips were precontracted with NA (3×10^{-6} M). Subsequently, frequency–response curves to electrical field stimulation (EFS) (1–32 Hz, pulse duration: 1 ms, pulse train: 10 s) of intrinsic inhibitory NANC nerves were constructed. In addition, concentration–response curves to NO, nitroglycerin (GTN), VIP, a VPAC1 receptor agonist (Gourlet et al., 1997a), a VPAC2 receptor agonist (Gourlet et al., 1997b), PACAP-27 and PACAP-38 were investigated. Relaxation to EFS was assessed in the absence and in the presence of the nitric oxide synthase (NOS) blocker L-nitroarginine (L-NNA, 3×10^{-4} M) and the nerve conductance blocker tetrodotoxin (TTX, 10^{-6} M). To study excitatory responses, experiments were performed on strips maintaining their basal tone. The contractile effect of galanin was investigated in the absence and in the presence of TTX (10^{-6} M) and of the α- and β-adrenoceptor blockers propranolol (10^{-6} M) and phentolamine (3×10^{-6} M). The incubation time of L-NNA, TTX, propranolol and phentolamine was 10 min.

Frequency–response curves and concentration–response curves were analysed by comparing their respective pD2 (the negative logarithm of the molar concentration inducing 50% of the maximal response) and E_{max} values.

2.2.3. Drugs used

The following drugs were used: atropine sulfate, guanethidine monosulfate, *N*ω-nitro-L-arginine (L-NNA), PACAP-27 (ovine isoform; A9808) and PACAP-38 (mammalian isoform; A1439; Sigma-Aldrich, St. Louis, MO, USA), phentolamine hydrochloride (Sigma-Aldrich), nitroglycerin (GTN), $NaNO_2$ (Merck, Darmstadt, Germany), propranolol hydrochloride (ICI Pharmaceuticals, Destelbergen, Belgium), galanin (porcine isoform; H-1365; Bachem/Peninsula Laboratories, San Carlos, USA), galantide (M15; Bachem/Peninsula Laboratories), L-noradrenalin-L-hydrogentartrat (Fluka, Switzerland), TTX (Alomone Labs, Jerusalem, Israel), VIP (synthetic porcine molecule; Bachem/Peninsula, St. Helens, USA), the VPAC1 agonist K^{15}, R^{16}, VIP(1–7), GRF(8–27)-NH_2 (Gourlet et al., 1997a), VPAC2 agonist (RO25-1553) (Gourlet et al., 1997b). All drugs were dissolved in distilled water. Solutions of NO were prepared as follows: a stock solution of 10^{-3} M $NaNO_2$ was prepared freshly in distilled water on the day of the experiment. Dilutions of the stock solution were made and, immediately before use, each solution was adjusted to pH 3 with 5×10^{-3} M HCl in order to generate NO from $NaNO_2$. A control solution of acidified distilled water did not induce relaxations in the muscle strips.

2.3. Presentation of results and statistical analysis

Contractions and relaxations were measured as the peak response to EFS or the agonist under study. Relaxations are expressed as % relaxation of the NA-induced contraction. Contractions are expressed in mN. All values are expressed as mean ± S.E.M. for the number (n) of hens indicated. For statistical analysis, Student's t-test for paired or unpaired observations was performed using SPSS software (Chicago, IL, USA). pD2 and E_{max} values were calculated by nonlinear regression fitting of the concentration–response curves using GraphPad Prism software (San Diego, CA, USA). $P < 0.05$ was considered as significantly different from controls.

3. Results

3.1. Immunohistochemistry

Single labelling revealed that galanin- and PACAP-27-immunoreactive (-IR) nerve bundle fibres ran between and innervated the scattered muscle bundles of both longitudinal and thick circular muscle layers (Figs. 1c–d and 2b). Galanin- and PACAP-27-IR perikarya were

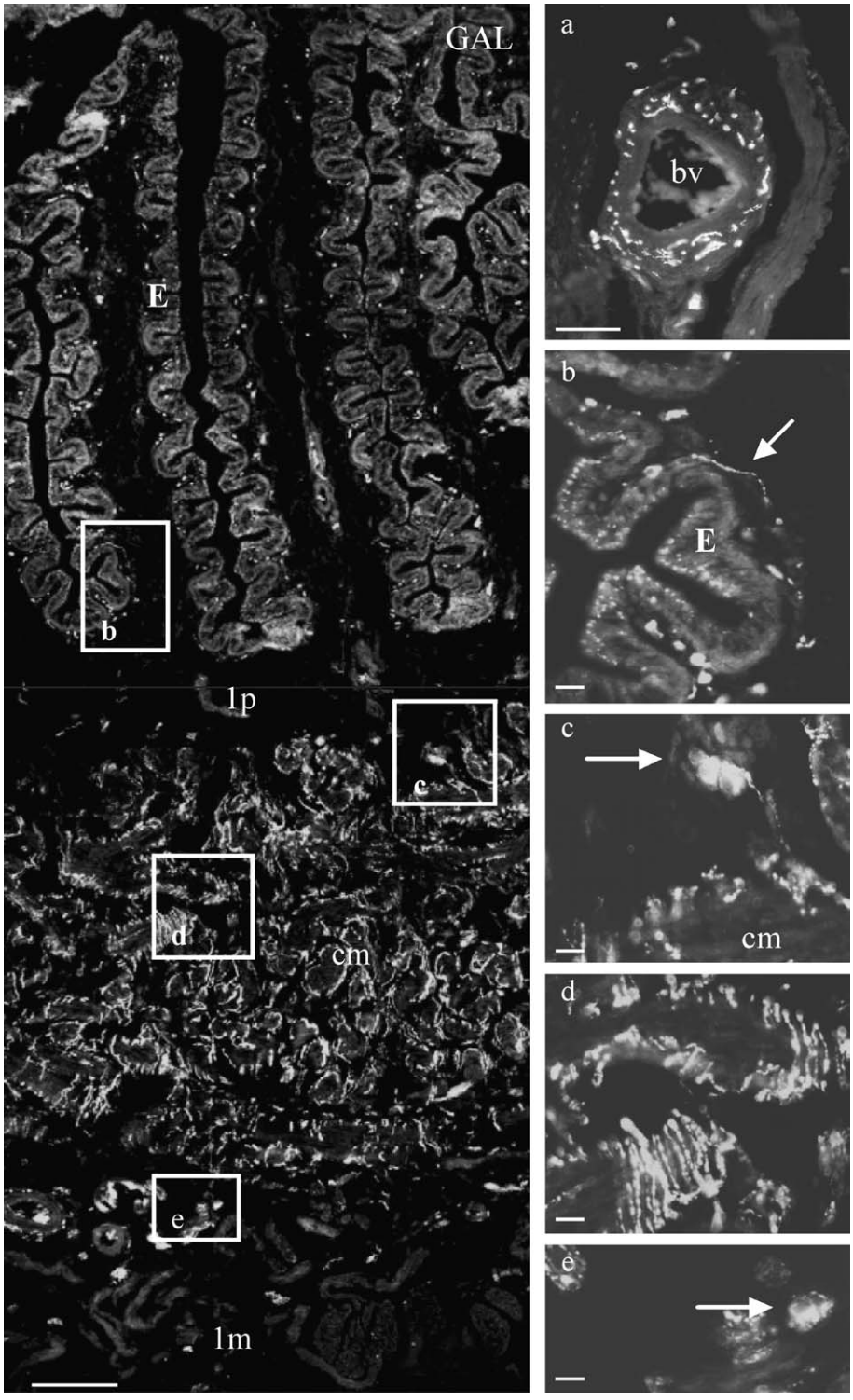

Fig. 1. Reconstruction of galanin (GAL)-containing nerves in the lower oviductal wall (vagina). Bar = 400 μm. Higher magnifications showing the presence of (a) perivascular galanin-IR nerve fibres in the mucosa (not indicated by a rectangle in the overview), (b) subepithelial IR nerve fibres (arrow). The fluorescence staining in the epithelial cells is unspecific, (c) two mucosal galanin-IR neurons projecting to the inner muscle bundle (arrow), (d) galanin-IR perimuscular nerve fibres, (e) a single galanin-IR neuron in the intermuscular space (arrow). (b–e) Higher magnifications of rectangles indicated in overview. Bar = 50 μm. lp: lamina propria, cm: circular muscle, lm: longitudinal muscle, bv: blood vessel, E: epithelium.

observed in the intermuscular space (Fig. 1e), between the muscle bundles of the circular muscle layer (Fig. 2b) and within the lamina propria (Fig. 1c). A few thin but distinct galanin- or PACAP-27-IR nerve fibres extended up into the mucosal folds, as such delineating the base of the epithelium (Figs. 1b and 2a). All IR neuronal pop-

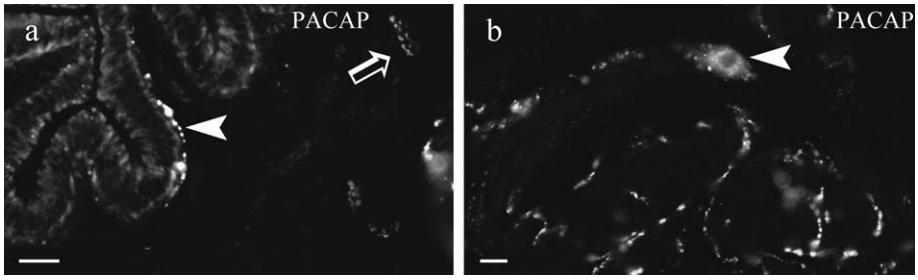

Fig. 2. PACAP-containing nerves in the lower oviduct (vagina). (a) Subepithelial IR nerve fibre (arrowhead) and scattered nerve fibres within the mucosal layer (arrow); (b) IR nerve fibres associated with muscle bundles and one IR nerve cell (arrowhead). Bar = 50 μm.

ulations displayed a similar distribution pattern. The intramural vascular net was also targeted by nerves IR for the above-mentioned peptides (Fig. 1a).

3.2. Multiple labelling for PACAP-27, galanin, VIP, NOS and tyrosine hydroxylase

Double labelling showed that PACAP-27 almost completely colocalised with VIP (Fig. 3a–a′) and NOS in both perikarya and fibres (Fig. 3c–c′ and d–d′). VIP-immunostaining occurred in the majority (75%) of the galanin-IR neurons (Fig. 3b–b′). NOS immunoreactivity was found in a large neuronal subpopulation containing galanin, and in neurons containing VIP, the latter confirming previous results (Costagliola et al., 1997). A small proportion of neurons IR for either VIP or galanin was also observed.

In contrast, TH (tyrosine hydroxylase)-IR nerve fibres innervated scattered muscle bundles without extending into the lamina propria, and contributed to the vascular innervation (Fig. 3e′); these fibres did not colocalise with either PACAP- or galanin-containing neurons. TH-IR perivascular nerve fibres were intertwined with peptidergic perivascular nerves, but no colocalisation was found (Fig. 3e–e′).

Triple labelling for PACAP, VIP and galanin confirmed that galanin colocalised with PACAP-27 and VIP in a large neuronal subpopulation (Fig. 3f–f″).

3.3. Pharmacology

3.3.1. Spontaneous activity

Spontaneous contractions of the vaginal strips occurred in all longitudinal and circular preparations at a high, constant frequency, although irregular patterns were also observed. NA produced TTX-insensitive excitatory effects. Addition of atropine and guanethidine as well as the α- and β-adrenergic blockers propranolol (10^{-6} M) and phentolamine (3×10^{-6} M) did not affect the pattern of spontaneous activity.

3.3.2. Effect of NANC nerve stimulation

In the presence of atropine and guanethidine and upon NA-induced contraction, EFS for 10 s at 1–32 Hz induced frequency-dependent relaxations of longitudinal and circular vaginal smooth muscle strips (Fig. 4). All relaxations to EFS, both in the circular and in the longitudinal muscle strips, were of neuronal origin since they were abolished by TTX (results not shown). L-NNA (3×10^{-4} M), a blocker of NO synthase, reduced the relaxations to EFS at low frequencies but had no effect on relaxations to higher frequency stimulation (Fig. 4).

3.3.3. Inhibitory role of PACAP-27 and PACAP-38, VPAC1 and VPAC2 agonists, NO and nitroglycerin

PACAP-27 and PACAP-38 (10^{-10}–3×10^{-7} M) concentration-dependently relaxed longitudinal and circular smooth muscle strips that were precontracted with NA (Fig. 5A–B). PACAP-27 was more potent than PACAP-38 in relaxing NA-precontracted muscle strips (Fig. 5A–B), which resulted in a significant difference in E_{max} but not in pD2 values (Table 2). Relaxations to PACAP-27 and PACAP-38 were not affected by TTX (10^{-6} M) or L-NNA (3×10^{-4} M) (results not shown).

Two different agonists for VIP receptors (VPAC1 and VPAC2) were used. The VPAC1 receptor agonist did not have a relaxant effect on NA-precontracted strips from longitudinal and circular oviductal smooth muscle (Fig. 6A). In contrast, the VPAC2 receptor agonist (VPAC2-ra) induced a dose-dependent relaxation of NA-precontracted muscle strips (Fig. 6B). E_{max} and pD2 values for VPAC2-ra did not differ between longitudinal and circular muscle strips (Table 2). The relaxations to VPAC2-ra were not affected by TTX or L-NNA in either the longitudinal or circular muscle strips (results not shown).

Both NO (Fig. 7A) and the intracellular NO-donor GTN (Fig. 7B) induced concentration-dependent relaxations of longitudinal and circular muscles from NA-precontracted vaginal strips. The E_{max} values for NO and GTN were comparable in longitudinal and circular muscle strips (Table 2). However, the pD2 values of GTN were significantly lower than those of NO (Table 2).

3.4. Excitatory role of galanin

Galanin caused a concentration-dependent contraction of the vaginal circular and longitudinal muscle strips (Fig. 8A) with an evident increase in the frequency and amplitude of the spontaneous contractions of the muscle strips. The pD2 value for galanin was comparable in longitudinal and

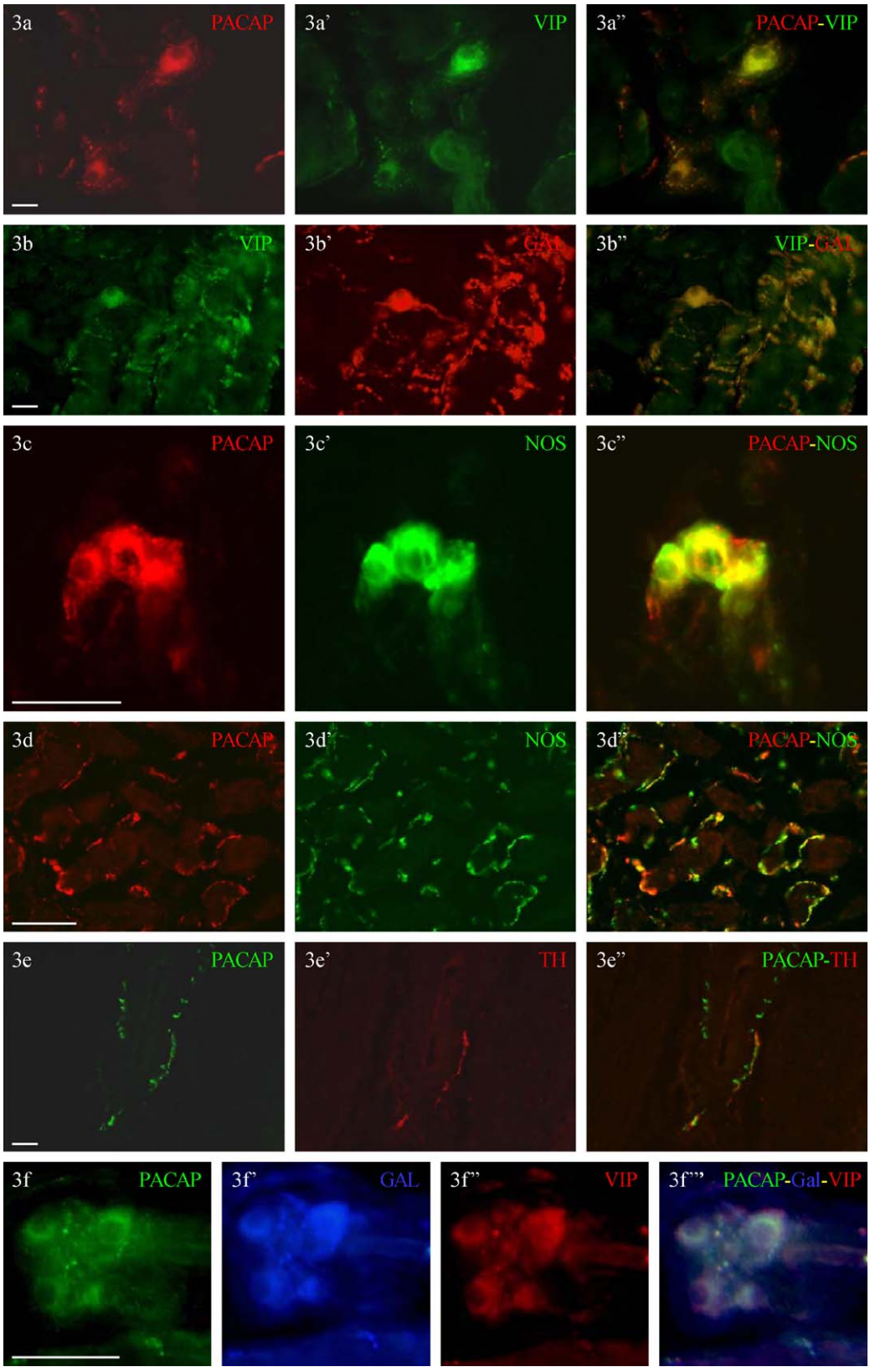

Fig. 3. Double labelling showing PACAP-containing neurons costoring VIP (a–a′) and NOS in both perikarya (c–c′) and nerve fibres (d–d′) within the muscle layer. VIP is costored in galanin (GAL)-containing neurons (b–b′). TH-IR perivascular nerves are intertwined with those expressing PACAP immunoreactivity, but no colocalisation was found (e–e′). Triple labelling shows PACAP, galanin (GAL) and VIP colocalised in the same neuronal population (f–f″). Bar = 50 μm, except (c–c′) = 100 μm.

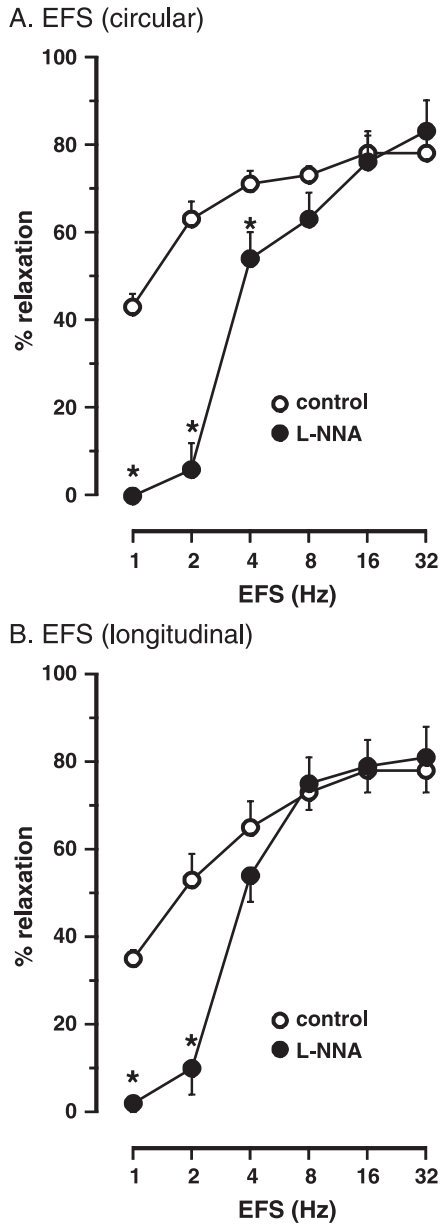

Fig. 4. Frequency–response curve to electrical field stimulation (EFS) of inhibitory nerves in circular (A) and longitudinal (B) muscle strips of the chicken oviduct and the effect of the NOS blocker L-NNA. Results are expressed as % relaxation of a noradrenaline-induced contraction and expressed as mean ± S.E.M. for $n=8$ experiments. *$P<0.05$, unpaired Student's t-test, significantly different from controls.

circular muscle strips (Table 2) but the E_{max} value was significantly lower in longitudinal strips (Table 2). The contractions to galanin were TTX-insensitive and were not affected by the α- and β-adrenergic blockers propranolol and phentolamine (Fig. 8B).

4. Discussion

Our results demonstrate the presence of galanin- and PACAP 27-IR nerve fibres and perikarya in the vaginal muscle wall of the egg-laying hen oviduct and show that galanin and PACAP exert a contractile and relaxant activity, respectively. This suggests that both substances are important regulators of motility in this region.

A previous study carried out on the oviduct of egg-laying hens (Costagliola et al., 1997) already reported the distribution of VIP/NOS-IR neurons along the entire oviductal length and showed significant regional differences. Throughout the oviductal canal, these NANC nerves mainly targeted the intramural vascular bed, whereas the vaginal segment harboured a dense nerve network containing intra-

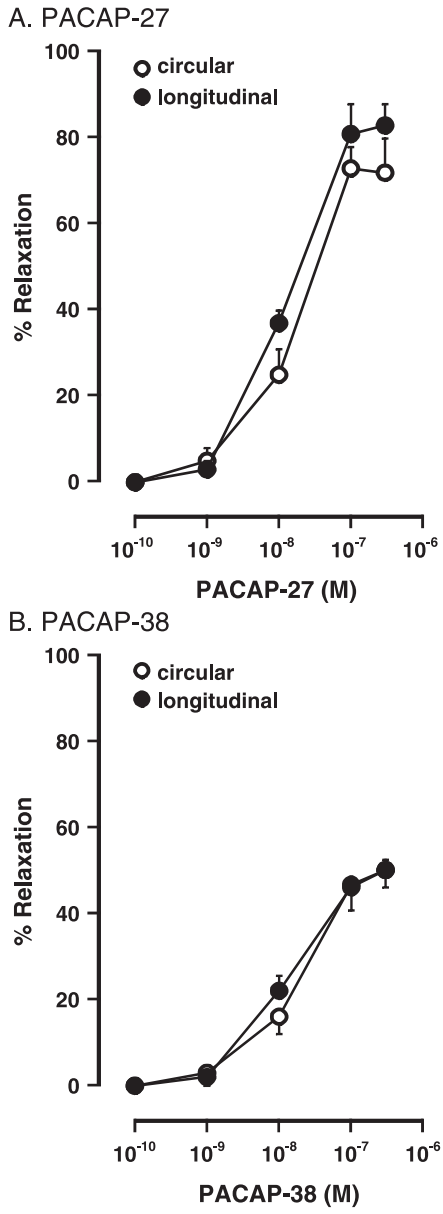

Fig. 5. Concentration–response curves to PACAP-27 (A) and PACAP-38 (B) in circular and longitudinal muscle strips of the chicken oviduct. Results are expressed as % relaxation of a noradrenaline-induced contraction and expressed as mean ± S.E.M. for $n=8$ experiments.

Table 2
Comparison of pD2 and E_{max} values for VPAC-2, PACAP-27, PACAP-38, NO, GTN and galanin in longitudinal and circular muscle strips of the chicken oviduct

	pD2		E_{max}	
	Longitudinal	Circular	Longitudinal	Circular
VPAC-2	8.09 ± 0.13	7.80 ± 0.08	93.4 ± 4.6%	90.9 ± 3.2%
PACAP-27	7.89 ± 0.11	7.68 ± 0.14	89.2 ± 4.1%	83.94 ± 4.9%
PACAP-38	7.87 ± 0.11	7.64 ± 0.18	53.5 ± 2.4%[a]	54.86 ± 4.1%[a]
NO	4.92 ± 0.12	4.69 ± 0.08	84.3 ± 3.9%	92.98 ± 2.9%
GTN	6.25 ± 0.09[b]	6.30 ± 0.12[b]	85.1 ± 2.4%	86.85 ± 3.4%
Galanin	8.22 ± 0.22	7.91 ± 0.23	3.35 ± 0.03 mN	5.69 ± 0.06 mN[c]

Results are expressed as mean ± S.E.M. (all $n = 6–8$).
[a] $p < 0.05$ significantly different from effect of PACAP-27 in corresponding muscle strips.
[b] $p < 0.05$ significantly different from effect of NO in corresponding muscle strips.
[c] $p < 0.05$ significantly different from effect of galanin in longitudinal muscle strips.

mural neuronal somata that were mainly located within the muscle layer, suggesting active involvement of VIP and NO in the mechanical activity of the vagina during oviposition. The similar distribution patterns seen in single stainings for galanin and PACAP together with the double and triple stainings confirmed that the vast majority of these intrinsic nerve fibres could be neurochemically identified as one population of VIP/galanin/PACAP/NOS-IR fibres. This population did not display immunoreactivity for TH and is hence clearly distinct from the postganglionic sympathetic fibres mainly targeting the intrinsic vascular supply of the segment. The exact nature of these fibres remains to be determined by tracing experiments, but the presence of a significant number of intramural cell bodies with identical neurochemical content makes it reasonable to assume an intrinsic origin.

The inhibitory role of PACAP, VIP and NO is now widely acknowledged, and these messenger molecules may coexist in the same neuronal subpopulation in different proportions depending on the species studied. Our study suggests that the N-terminal region of PACAP (PACAP-27) is the biologically active substance in this avian tissue, which is in accordance with the dense PACAP-27 nerve supply to the muscle observed in this study. PACAP can exert its actions via the activation of at least three types of receptors with different affinities (PAC1, VPAC1, VPAC2; Harmar et al., 1998; Robberecht et al., 1998), which belong to a subfamily of the seven transmembrane-spanning G protein-coupled receptors. Some of these mammalian receptors (e.g. the PAC1 receptor) have been cloned in birds as well (Peeters et al., 1999). PACAP-27 may link with higher affinity ($K_d \sim 0.5$ nM) than VIP (K_d>500 nM) to its specific PAC1 receptor (Harmar et al., 1998), whereas the VPAC1 and VPAC2 receptors display similar affinities for PACAP and VIP ($K_d \sim 1$ nM). In the present study PACAP-27 and PACAP-38 were both found to induce a relaxation of isolated vaginal muscle strips, with PACAP-27 being more potent than PACAP-38. These relaxations were not affected by TTX suggesting a direct action of PACAP on smooth muscle cells. VIP was previously found to inhibit vaginal motility during oviposition (Houvenaghel and Wechsung, 1989). This study clearly demonstrates that VIP is able to directly induce relaxation of the vaginal smooth muscle and that this relaxant effect is mimicked by a VPAC2 receptor agonist but not by a VPAC1 receptor agonist. Taken together, the present results suggest that the VIP and PACAP effects in the hen vagina are mediated through VPAC2 receptors. The observed VIP-induced relaxation is likely caused via activation of a cyclic AMP-dependent pathway (Bolton, 1979) and not via generation of NO since L-NNA has no effect on the VPAC2 agonist-induced relaxations.

In the hen vagina, VIP-IR was found to be colocalised with nitrergic neurons also costoring PACAP in a large neuronal population (Costagliola et al., 1997; present paper). The present study provides functional evidence that NO is involved in the inhibitory neurotransmission in the vaginal segment. Firstly, both NO and the NO donor GTN were able to relax precontracted circular and longitudinal muscle strips. Secondly, EFS of intramural nerves in these oviductal muscle strips induced frequency-dependent relaxations. The relaxations to low frequency stimulation were abolished by L-NNA, suggesting that NO was the sole mediator of these relaxations. Interestingly, the relaxations to higher frequency stimulation were not affected by L-NNA. This finding points to possible involvement of other neurotransmitters in inhibitory NANC neurotransmission of the vaginal region in the hen oviduct.

Since PACAP, VIP and NO coexist with galanin in intramural neurons of the hen vagina, we also investigated the role of galanin in oviductal neurotransmission. The hen differs in this respect from other avian models, such as quail and pigeon, which do exhibit galanin-IR nerve fibres but not cell bodies in their oviductal wall (Li et al., 1996; Atoji et al., 2000). In mammals, galanin has been reported to be colocalised with VIP and NOS in the NANC innervation of several organs (Furness et al., 1992; Song et al., 1992; Zhu and Dey, 1992; Timmermans et al., 1994a,b; Lakomy et al., 1995; Vittoria et al., 2000). Galanin has further been reported to produce opposite effects (Delvaux et al., 1991; Botella et al., 1992, 1995) attributable to the involvement of different receptors. To

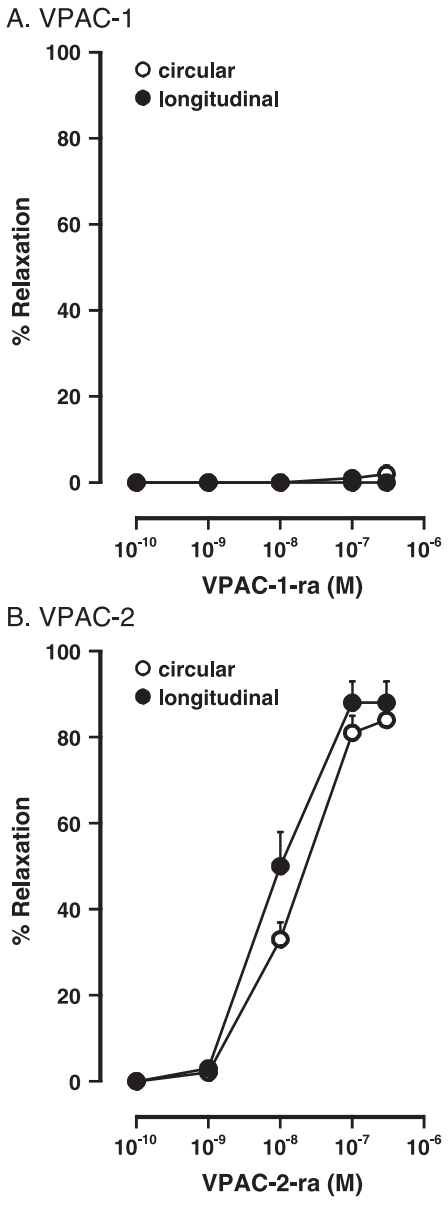

Fig. 6. Concentration–response curves to receptor agonists of VPAC1 (VPAC1-ra) (A) and VPAC2 (VPAC2-ra) (B) in circular and longitudinal muscle strips of the chicken oviduct. Results are expressed as % relaxation of a noradrenaline-induced contraction and expressed as mean ± S.E.M. for $n = 8$ experiments.

date, three different subtypes of galanin receptors have been cloned in mammals that belong to a family of G-protein-coupled receptors. They show a different tissue distribution and activate different second messenger pathways (for review, see Vrontakis, 2002). In the vaginal part of the hen oviduct, galanin is costored and released by inhibitory motor neurons to vaginal muscle bundles (present paper). Our pharmacological data revealed excitatory effects of galanin that were TTX-insensitive. The contractions in response to galanin application were not affected after blockade of α- and β-adrenoceptors, suggesting that the contractile effect of galanin is a direct action on the smooth muscle and is not mediated via adrenergic pathways. These findings corroborate the results reported in the quail oviduct (Li et al., 1996) and chicken gut (DeGolier et al., 1999), although in those experiments it cannot be ruled out that the contractile effects produced by galanin might be attributed to a neurogenic action next to myogenic activity in these species.

In conclusion, galanin, PACAP-27, VIP and NO are NANC neurotransmitters that are found in a large intrinsic neuronal subpopulation in the chicken vaginal wall. These NANC neurotransmitters are able to contract (galanin) and relax (PACAP, VIP, NO) the vaginal smooth muscle wall

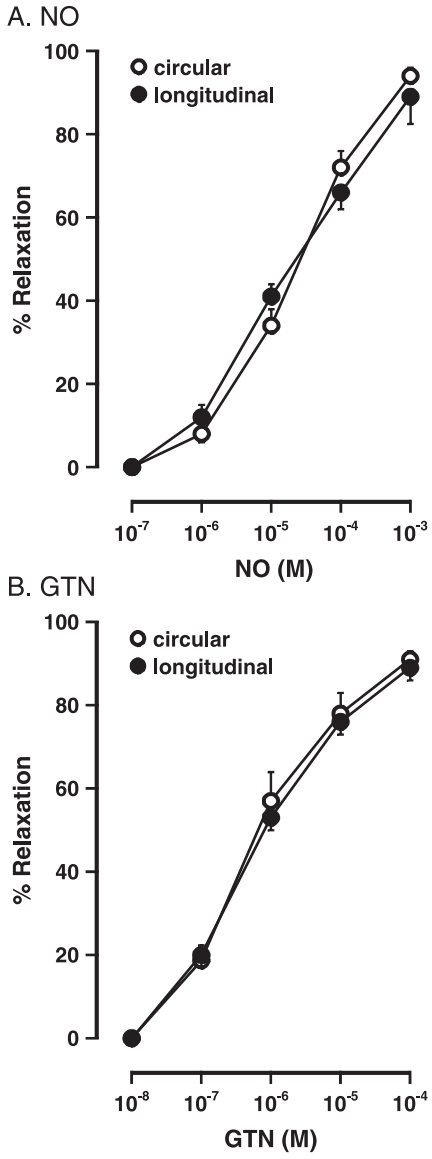

Fig. 7. Concentration–response curves to NO (A) and nitroglycerin (GTN) (B), a NO donor, in circular and longitudinal muscle strips from the chicken oviduct. Results are expressed as % relaxation of a noradrenaline-induced contraction and expressed as mean ± S.E.M. for $n = 8$ experiments.

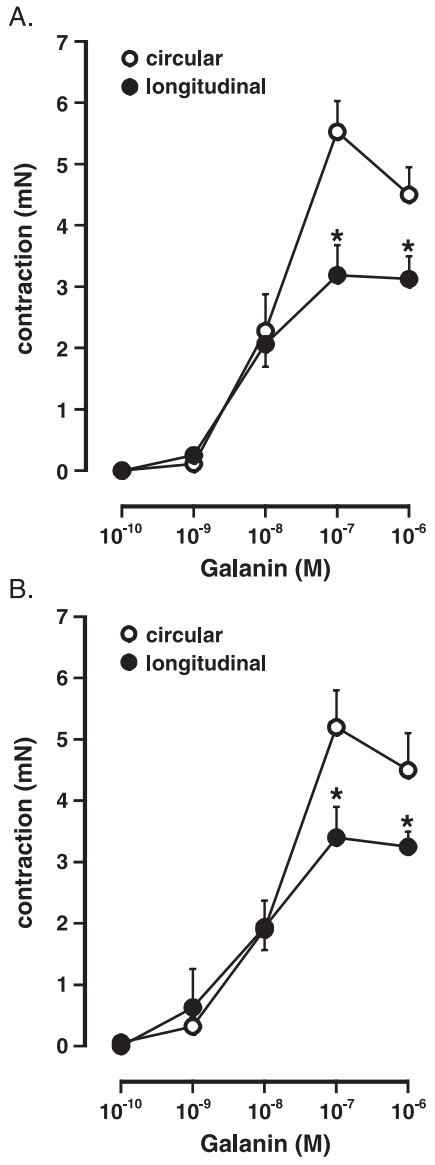

Fig. 8. Concentration–response curves to galanin under control conditions (A) and in the presence of propranolol and phentolamine (B) in circular and longitudinal muscle strips of the chicken oviduct. Results are expressed as % relaxation of a noradrenaline-induced contraction and expressed as mean ± S.E.M. for $n=8$ experiments. *$P<0.05$, unpaired Student's t-test, significantly different from controls.

by a direct myogenic action. This suggests that they are important mediators of muscle activity in this part of the chicken oviduct and play a role in the process of oviposition.

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References

Ahren, B., Bottcher, G., Kowalyk, S., Dunning, B.E., Sundler, F., Taborsky, G.J., 1990. Galanin is co-localized with noradrenalin and neuropeptide Y in dog pancreas and celiac ganglion. Cell Tissue Res. 261, 49–58.

Atoji, Y., Mizutani, K., Yamamoto, Y., Suzuki, Y., 2000. Innervation of the pigeon oviduct: correlation of NADPH diaphorase with acetylcholinesterase, tyrosine hydroxylase, and neuropeptides. Auton. Neurosci. 84, 1–7.

Barnes, P.J., 1987. Regulatory peptides in the respiratory system. Experientia 43, 832–839.

Bauer, F.E., 1990. Galanin: a new biologically active gastrointestinal neuropeptide. Z. Gastroenterol. 28, 160–169.

Bauer, F.E., Christofides, N.D., Hacker, G.W., Blank, M.A., Polak, J.M., Bloom, S.R., 1986. Distribution of galanin immunoreactivity in the genito-urinary tract of man and rat. Peptides 7, 5–10.

Bennett, T., Malmfors, T., Cobb, J.L.S., 1973. A fluorescence histochemical study of the degeneration and regeneration of noradrenergic nerves in the chick following treatment with 6-hydroxydopamine. Z. Zellforsch. 142, 103–130.

Bolton, T.B., 1979. Mechanisms of action of transmitters and other substances on smooth muscle. Phys. Rev. 59, 606–718.

Botella, A., Delvaux, M., Frexinos, J., Bueno, L., 1992. Comparative effects of galanin on isolated smooth muscle cells from ileum in five mammalian species. Life Sci. 50, 1253–1261.

Botella, A., Delvaux, M., Fioramonti, J., Frexinos, J., Bueno, L., 1995. Galanin contracts and relaxes guinea pig and canine intestinal smooth muscle cells through distinct receptors. Gastroenterology 108, 3–11.

Brouns, I., Van Nassauw, L., Van Genechten, J., Majewski, M., Scheuermann, D.W., Timmermans, J.-P., Adriaensen, D., 2002. Triple immunofluorescence staining with antibodies raised in the same species to study the complex innervation pattern of intrapulmonary chemoreceptors. J. Histochem. Cytochem. 50, 575–582.

Brouns, I., Pintelon, I., Van Genechten, J., De Proost, I., Timmermans, J.-P., Adriaensen, D., 2004. Vesicular glutamate transporter 2 is expressed in different nerve fibre populations that selectively contact pulmonary neuroepithelial bodies. Histochem. Cell Biol. 121, 1–12.

Costagliola, A., Mayer, B., Vittoria, A., Carrese, E., Lamanna, C., Cecio, A., 1997. NADPH-diaphorase, nitric oxide synthase- and VIP-containing nerve structures in the hen oviduct: a histochemical and immunohistochemical study. Arch. Histol. Cytol. 60, 245–256.

Crossley, J., Ferrando, G., Eiler, H., 1980. Distribution of adrenergic receptors in the domestic fowl oviduct. Poultry Sci. 59, 2331–2335.

DeGolier, T.F., Nordell, J.N., Pust, M.H., Duke, G.E., 1999. Effect of galanin on isolated strips of smooth muscle from the gastrointestinal tract of chickens. J. Comp. Exp. Zool. 283, 463–468.

Delvaux, M., Botella, A., Fioramonti, J., Frexinos, J., Bueno, L., 1991. Galanin induces contraction of isolated cells from circular muscle layer of pig ileum. Regul. Pept. 32, 369–374.

Doyle, L.L., Margolis, A.J., 1963. Intrauterine foreign body, effect on pregnancy in the rat. Science 139, 833.

Ekblad, E., Håkanson, R., Sundler, F., Wahlestedt, C., 1985. Galanin: neuromodulatory and direct contractile effects on smooth muscle preparations. Br. J. Pharmacol. 86, 241–246.

Furness, J.B., Bornstein, J.C., Murphy, R., Pompolo, S., 1992. Roles of peptides in transmission in the enteric nervous system. Trends Neurosci. 15, 66–71.

Gilbert, A.B., Lake, P.E., 1963. Terminal innervation of the uterus and vagina of the domestic hen. J. Reprod. Fertil. 5, 41–48.

Gourlet, P., Vandermeers, A., Vertongen, P., Rathe, J., De Neef, P., Cnudde, J., Waelbroeck, M., Robberecht, P., 1997a. Development of high affinity selective VIP_1 receptor agonists. Peptides 18, 1539–1545.

Gourlet, P., Vertongen, P., Vandermeers, A., Vandermeers-Piret, M.-C., Rathe, J., De Neef, P., Waelbroeck, M., Robberecht, P., 1997b. The long-acting vasoactive intestinal polypeptide agonist RO 25-1553 is highly selective of the VIP_2 receptor subclass. Peptides 18, 403–408.

Harmar, A.J., Arimura, A., Gozes, I., Journot, L., Laburthe, M., Pisegna, J.R., Rawlings, S.R., Robberecht, P., Said, S.I., Sreedharan, S.P., Wank, S.A., Waschek, J.A., 1998. International union of pharmacology: XVIII. Nomenclature of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide. Pharmacol. Rev. 50, 265–270.

Houvenaghel, A., Wechsung, E., 1989. Effects of prostaglandins, leukotrienes, VIP, carbachol and isopropylnoradrenaline on uterine and vaginal motility in the chicken. Verh. K. Acad. Geneeskd. Belg. 51, 153–174.

Hoyle, C.H.V., 1998. Neuropeptide families: evolutionary perspectives. Reg. Pept. 73, 1–33.

Hoyle, C.H.V., Burnstock, G., 1989. Galanin-like immunoreactivity in enteric neurons of the human colon. J. Anat. 166, 23–33.

Jozsa, R., Mess, B., 1993. Galanin-like immunoreactivity in the chicken brain. Cell Tissue Res. 273, 391–399.

Kaplan, L.M., Spindel, E.R., Isselbacher, K.J., Chin, W.W., 1988. Tissue-specific expression of the rat galanin gene. Proc. Natl. Acad. Sci. U. S. A. 85, 1065–1069.

Koike, T.I., Shimada, K., Cornett, L.E., 1988. Plasma levels of immunoreactive mesotocin and vasotocin during oviposition in chickens: relationship to oxytocic action of the peptides in vitro and peptide interaction with myometrial membrane binding sites. Gen. Comp. Endocrinol. 70, 119–126.

Lake, P.E., Gilbert, A.B., 1964. The effect on egg production of a foreign object in the lower oviduct regions of the domestic hen. Res. Vet. Sci. 5, 39–45.

Lakomy, M., Kaleczyc, J., Majewski, M., Sienkiewicz, W., 1995. Immunohistochemical localization of galanin in the bovine reproductive organs. Anat. Histol. Embryol. 24, 251–256.

Li, D., Tsutsui, K., Muneoka, Y., Minakata, H., Nomoto, K., 1996. An oviposition-inducing peptide: isolation, localization, and function of avian galanin in the quail oviduct. Endocrinology 137, 1618–1626.

Lindh, B., Lundberg, J.M., Hökfelt, T., 1989. NPY-, galanin-, VIP/PHI-, CGRP- and substance P-immunoreactive neuronal subpopulations in cat autonomic and sensory ganglia and their projections. Cell Tissue Res. 256, 259–273.

Luts, A., Uddman, R., Sundler, F., 1989. Neuronal galanin is widely distributed in the chicken respiratory tract and coexists with multiple neuropeptides. Cell Tissue Res. 256, 95–103.

Miyata, A., Arimura, A., Dahl, R.R., Minamino, N., Uehara, A., Jiang, L., Culler, M.D., Coy, D.H., 1989. Isolation of a novel 38 residue-hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells. Biochem. Biophys. Res. Commun. 164, 567–574.

Norberg, A., Sillard, R., Carlquist, M., Jornvall, H., Mutt, V., 1991. Chemical detection of natural peptides by specific structures. Isolation of chicken galanin by monitoring for its N-terminal dipeptide, and determination of the amino acid sequence. FEBS Lett. 288, 151–153.

Parsons, R.L., Neel, D.S., Konopka, L.M., McKeon, T.W., 1989. The presence and possible role of a galanin-like peptide in the mudpuppy heart. Peptides 29, 749–759.

Peeters, K., Berghman, L.R., Vandesande, F., 1998. Comparative distribution of pituitary adenylate cyclase-activating polypeptide and vasoactive intestinal polypeptide immunoreactivity in the chicken forebrain. Ann. N.Y. Acad. Sci. 15, 417–419.

Peeters, K., Gerets, H.H.J., Princen, K., Vandesande, F., 1999. Molecular cloning and expression of a chicken pituitary adenylate cyclase-activating polypeptide receptor. Mol. Brain Res. 71, 244–255.

Robberecht, P., De Neef, P., Lefebvre, R.A., 1998. Influence of selective VIP receptor agonists in the rat gastric fundus. Eur. J. Pharmacol. 359 (1), 77–80.

Rokäeus, Å., Carlquist, M., 1988. Nucleotide sequence analysis of cDNAs encoding a bovine galanin precursor protein in the adrenal medulla and chemical isolation of bovine gut galanin. FEBS Lett. 234, 400–406.

Rzasa, J., Ewy, Z., 1970. Effect of vasotocin and oxytocin on oviposition in the hen. J. Reprod. Fertil. 21, 549–550.

Salvi, E., Vaccaro, R., Renda, T.G., 1999. Ontogeny of galanin-immunoreactive elements in the intrinsic nervous system of the chicken gut. Anat. Rec. 197, 283–291.

Schmidt, W.E., Kratzin, H., EcKart, K., Drevs, D., Mundkowski, G., Clemens, A., Katsoulis, S., Schafer, H., Gallwitz, B., Creutzfeldt, W., 1991. Isolation of primary structure of pituitary human galanin, a 30-residue nonamidated neuropeptide. Proc. Natl. Acad. Sci. U. S. A. 88, 11435–11439.

Song, Z.-M., Brookes, S.J.H., Steele, P.A., Costa, M., 1992. Projections and pathways of submucous neurons to the mucosa of the guinea-pig small intestine. Cell Tissue Res. 269, 87–98.

Stjernquist, M., Ekblad, E., Owman, C., Sundler, F., 1988. Immunocytochemical localization of galanin in the rat male and female genital tracts and motor effects in vitro. Regul. Pept. 20, 335–343.

Sundler, F., Ekblad, E., Absood, A., Håkanson, R., Köves, K., Arimura, A., 1992. Pituitary adenylate cyclase activating polypeptide: a novel vasoactive intestinal peptide-like neuropeptide in the gut. Neuroscience 46, 439–454.

Takahashi, T., Kawashima, M., Kamiyoshi, M., Tanaka, K., 1992. Arginine vasotocin binding component in the uterus (shell gland) of the chicken. Acta Endocrinol. 127, 179–184.

Takahashi, T., Kawashima, M., Yasuota, T., Tanaka, K., 1998. Arginine vasotocin receptor in the vagina of the oviduct of the hen. Poultry Sci. 77 (11), 1699–1703.

Tatemoto, K., Rokäeus, Å., Jornvall, H., McDonald, T.J., Mutt, V., 1983. Galanin—a novel biologically active peptide from porcine intestine. FEBS Lett. 164, 124–128.

Timmermans, J.-P., Barbiers, M., Scheuermann, D.W., Stach, W., Adriaensen, D., Mayer, B., De Groodt-Lasseel, M.H., 1994a. Distribution pattern, neurochemical features and projections of nitrergic neurons in the pig small intestine. Anat. Anz. 176, 515–525.

Timmermans, J.-P., Barbiers, M., Scheuermann, D.W., Bogers, J.J., Adriaensen, D., Fekete, E., Mayer, B., Van Marck, E., De Groodt-Lasseel, M.H.A., 1994b. Nitric oxide-synthase immunoreactivity in the enteric nervous system of the developing human digestive tract. Cell Tissue Res. 275, 235–245.

Tsutsui, K., Li, D., Azumaya, Y., Muneoka, Y., Minakata, H., Nomoto, K., 1997. Demonstration, localization, and development of galanin receptors in the quail oviduct. J. Exp. Zool. 277, 57–65.

Ulman, L.G., Evans, H.F., Iismaa, T.P., Potter, E.K., McCloskey, D.I., Shine, J., 1992. Effects of human, rat and porcine galanins on cardiac vagal action and blood pressure in the anaesthetised cat. Neurosci. Lett. 136, 105–108.

Vaudry, D., Gonzales, B.J., Basille, M., Yon, L., Fournier, A., Vaudry, H., 2000. Pituitary adenylate cyclase-activating polypeptide and its receptors: from structure to functions. Pharmacol. Rev. 52, 269–324.

Verma, O.P., Prasad, B.K., Yancey, A., 1977. Adrenergic activity of the oviduct of the domestic fowl. J. Reprod. Fertil. 49, 195–200.

Vittoria, A., Costagliola, A., Carrese, E., Mayer, B., Cecio, A., 2000. Nitric oxide containing neurons in the bovine gut, with special reference to their relationship with VIP and galanin. Arch. Histol. Cytol. 63 (4), 357–368.

Vrontakis, M.E., 2002. Galanin: a biologically active peptide. Curr. Drug Targets—CNS Neurol. Dis. 1, 531–541.

Wechsung, E., Houvenaghel, A., 1976. A possible role of prostaglandins in the regulation of ovum transport and oviposition in the domestic hen. Prostaglandins 2, 269–279.

Zhu, W., Dey, R.D., 1992. Distribution of the neuropeptide galanin in the cat heart and coexistence with vasoactive intestinal peptide, substance P and neuropeptide Y. J. Mol. Cell. Cardiol. 24, 35–41.