Double labelling immunohistochemical characterization of autonomic sympathetic neurons innervating the sow retractor clitoridis muscle

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The retractor clitoridis muscle (RCM), absent in primates, dogs and some rodents, is a paired band of smooth muscle that in the sow originates directly from the smooth muscle of the anus and terminates at the base of the clitoris. Differently from the other smooth muscles of genital organs, it does not form one of their layers, nor is it a component of their fibromuscular stroma. Moreover, the RCM is in tonic contraction in the rest phase and relaxed in the active phase. This peculiarity makes it an interesting experimental model.

The present study documents the complexity of the neurochemical interactions that regulate the activity of the smooth myocytes of the RCM and their vascular components.

Key words: immunohistochemistry, retrograde tracing, peripheral autonomic neurons, genital smooth muscle, vascular smooth muscle, pig.

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aimed at defining, within the neurons of the S1 paravertebral ganglion (PaG S1), (shown to contain the highest number of sympathetic neurons supplying the muscle (Panu et al., 2001)), the pattern and the percentage of co-existence of TH, with: 1 choline acetyltransferase (ChAT), a marker of cholinergic neurons, 2, neuronal nitric oxide synthase (nNOS), a marker of nitric oxide, and 3, one of the following biologically active peptides, i.e. calcitonin gene related peptide (CGRP), leucine-enkephalin (LENK), neuropeptide Y (NPY), substance P (SP) and vasoactive intestinal peptide (VIP).

This study was carried out in the swine a species which is frequently used as a research model (Kaleczyc et al., 1993, 1997; Majewski et al., 1995, 1999).

Preliminary data of this investigation have been published in abstract form (Bo Minelli et al., 2002b).

Materials and Methods

All procedures were approved by the local Ethics Committee for Animal Experimentation and by the Italian Ministry of Health. Precautions aimed at avoiding unnecessary suffering were taken at all stages of the experiment.

The study was carried out on the RCM of three 50 kg sows combining the retrograde neuronal tracer FB and double labelling immunofluorescence techniques.

The central part of the left RCM of each animal, under general anaesthesia, was inoculated with 50 µl of 2% w/v FB, a fluorescent tracer with cytoplasmatic affinity, by the use of a Hamilton syringe.

After a 7-day survival time, the animals, under general anaesthesia, were intracardially perfused, first with heparinized physiological solution and afterwards with fixative solution (4% w/v paraformaldehyde in 0.1 M phosphate buffer (PBS), pH 7.4). Before collecting the samples, macroscopic and microscopic examination of the RCM and adjacent tissues revealed that the distribution of FB was confined to the muscle and no evidence of the tracer in the surrounding tissues was found. This confirmed that the fascial connective tissue of the muscle represents a barrier capable of preventing the dispersion of the tracer in the surrounding tissues.

The ipsilateral PaG S1 was removed from each animal, post-fixed by immersion in the same fixative for 2 hr at 4°C, rinsed with PBS, pH 7.4 and transferred into a 10% w/v buffered sucrose solution (pH 7.4) for 24 h. Afterwards, they were transferred into a 30% w/v buffered sucrose solution (pH 7.4) where they were stored at 4°C for at least three days or until further processing.

Each ganglion was placed flat in the cryostat mould and serially cut along its longest axis, in order to obtain a large number of cells per section.

The 12 µm thick sections were stained by a double labelling immunofluorescence method to test the occurrence and co-localization of TH with ChAT, nNOS, CGRP, LENK, NPY, SP and VIP.

The same combinations of primary antisera were applied to sections at least 96 µm away from each other to eliminate the likelihood of testing the same neuron twice for the same antisera. After air-drying at room temperature (rt) for 30 min, the sections were incubated with a solution containing 0.25% Triton X-100, 1% bovine serum albumin and 10% normal goat serum in PBS for 1 hr (rt), to reduce non-specific background staining. They were then incubated with the combination of primary antisera (overnight, rt) and, finally, incubated with Texas Red-conjugated streptavidin (primary antisera and secondary reagents are listed in Table 1) and mounted in buffered glycerin (Bio-Optica). Each step of immunolabelling was followed by rinsing the sections with PBS (3 × 5 min; pH 7.4). In control experiments, no immunoreactivity was detected in sections incubated in the absence of primary antisera, replaced by PBS, and in sections incubated with the rabbit antisera which had been adsorbed with excess (100 µg/mL) of the respective antigens, when these were available [rat CGRP (Sigma), porcine NPY (Sigma), synthetic VIP (Sigma)].

The labelled sections were studied and photographed with a Zeiss Axioskop 2 plus fluorescence microscope equipped with epi-illumination and appropriate filter for FB (excitation wavelength 390-420 nm; emission wavelength 450 nm), FITC (excitation wavelength 450-490 nm; emission wavelength 515-565 nm) and Texas Red (excitation wavelength 530-585 nm; emission wavelength 615 nm). Relationships between FB distribu-
tion and immunohistochemical staining were examined directly by interchanging filters. The observations were made by a single operator.

In each ganglion, the relative percentages of RCM-projecting neurons containing different combinations of the markers were calculated on the total number of FB-labelled (FB+) cells tested for each couple of primary antisera. Data are expressed as means ± S.E.M.

Results

Each combination of primary antisera was tested on a mean number of 179.05±12.93 FB+ neurons of the PaG S1 from each animal. 57.91±10.03% of the FB+ neurons showed TH-IR. The immunofluorescence varied from strong to moderate and was uniformly distributed through the perikarya, sometimes also being evident along the neuronal processes.

Double labelling immunofluorescence showed that TH was co-localized in different proportions with each one of the other tested substances (Table 2).

Table 1. Antisera and dilutions used in the experiments.

<table>
<thead>
<tr>
<th>Primary antisera</th>
<th>Raised in</th>
<th>Code no.</th>
<th>Supplier</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-tyrosine hydroxylase (TH)</td>
<td>mouse (monoclonal)</td>
<td>T 2928</td>
<td>Sigma, St. Louis, Missouri, U.S.A.</td>
<td>1:4000</td>
</tr>
<tr>
<td>Anti-choline acetyltransferase (ChAT)</td>
<td>rabbit (polyclonal)</td>
<td>AB 5042</td>
<td>Chemicon International, Inc., Temecula, CA</td>
<td>1:3000</td>
</tr>
<tr>
<td>Anti-neuronal nitric oxide synthase (n-NOS)</td>
<td>rabbit (polyclonal)</td>
<td>AB 5380</td>
<td>Chemicon International, Inc., Temecula, CA</td>
<td>1:1500</td>
</tr>
<tr>
<td>Anti-calcitonin gene related peptide (CGRP)</td>
<td>rabbit (polyclonal)</td>
<td>C 8198</td>
<td>Sigma, St. Louis, Missouri, U.S.A.</td>
<td>1:4000</td>
</tr>
<tr>
<td>Anti-leu-enkephalin (LENK)</td>
<td>rabbit (polyclonal)</td>
<td>L 8516</td>
<td>Sigma, St. Louis, Missouri, U.S.A.</td>
<td>1:5</td>
</tr>
<tr>
<td>Anti-neuropeptide Y (NPY)</td>
<td>rabbit (polyclonal)</td>
<td>N 9528</td>
<td>Sigma, St. Louis, Missouri, U.S.A.</td>
<td>1:4000</td>
</tr>
<tr>
<td>Anti-substance P (SP)</td>
<td>rabbit (polyclonal)</td>
<td>S 1542</td>
<td>Sigma, St. Louis, Missouri, U.S.A.</td>
<td>1:4000</td>
</tr>
<tr>
<td>Anti-vasoactive intestinal polypeptide (VIP)</td>
<td>rabbit (polyclonal)</td>
<td>V 3508</td>
<td>Sigma, St. Louis, Missouri, U.S.A.</td>
<td>1:4000</td>
</tr>
</tbody>
</table>

Secondary antisera

| Anti rabbit IgG/FITC                  | goat               | F 0382   | Sigma, St. Louis, Missouri, U.S.A. | 1:40 |
| Anti mouse IgG/biotin                 | sheep              | RPN 1001 | Amersham Pharmacia Biotech, U.K.   | 1:100 |
| Streptavidin/Texas red                |                    | RPN 1233 | Amersham Pharmacia Biotech, U.K.   | 1:100 |

Table 2. Number (No.) of RPM-projecting (FB+) cells tested for each pair of primary antisera and relative percentages (RP) of FB+ neurons showing different combination of positivity to each pair of tested primary antisera. Data are expressed as means ± S.E.M.

<table>
<thead>
<tr>
<th>Tested combination of primary antisera</th>
<th>TH/Ab (RP)</th>
<th>TH/Ab (RP)</th>
<th>TH/Ab (RP)</th>
<th>TH/Ab (RP)</th>
<th>FB tested cells (No.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TH-CGRP</td>
<td>3.1±0.174</td>
<td>63.91±7.333</td>
<td>0.34±0.18</td>
<td>32.93±8.99</td>
<td>173.33±38.85</td>
</tr>
<tr>
<td>TH-LENK</td>
<td>4.49±2.86</td>
<td>55.69±6.11</td>
<td>1.68±1.45</td>
<td>37.36±5.77</td>
<td>210.67±54.77</td>
</tr>
<tr>
<td>TH-nNOS</td>
<td>6.90±3.18</td>
<td>49.92±11.36</td>
<td>2.53±1.34</td>
<td>40.65±11.95</td>
<td>185.33±40.34</td>
</tr>
<tr>
<td>TH-NPY</td>
<td>3.14±0.63</td>
<td>28.29±2.92</td>
<td>11.10±1.71</td>
<td>26.49±10.76</td>
<td>167.30±70.75</td>
</tr>
<tr>
<td>TH-SP</td>
<td>6.76±5.11</td>
<td>54.33±5.37</td>
<td>0.00±0.00</td>
<td>38.91±5.53</td>
<td>178±11.02</td>
</tr>
<tr>
<td>TH-ChAT</td>
<td>4.28±2.16</td>
<td>55.49±5.85</td>
<td>0.55±0.37</td>
<td>39.68±7.92</td>
<td>179±39.25</td>
</tr>
<tr>
<td>TH-VIP</td>
<td>2.48±2.48</td>
<td>49.54±10.66</td>
<td>0.00±0.00</td>
<td>47.98±13.14</td>
<td>160±24.56</td>
</tr>
</tbody>
</table>
Figure 1. Fluorescence micrographs of PaG S1 FB+ cells showing positivity to the different couples of tested antisera. Respectively: A= a FB+ cell (A1) showing also positivity to TH (A2) and NPY (A3); B= a FB+ cell (B1) showing also positivity to TH (B2) and nNOS (B3); C= a FB+ cell (C1) showing also positivity to TH (C2) and SP (C3); D= a FB+ cell (D1) showing also positivity to TH (D2) and LENK (D3); E= three FB+ cells (E1) showing also positivity to TH (E2) and ChAT (E3); F= a FB+ cell (F1) showing also positivity to TH (F2) and CGRP (F3); G= a FB+ cell (G1) showing also positivity to TH (G2) and VIP (G3). Scale bar = 50 µm. Magnification, 40X.
jects, as was that of the TH and CGRP co-localization (3.10±1.74%, n=173.33±38.85; Figure 1, F1-F3); while the co-existence of TH and VIP was detected in only one subject (2.48±2.48%, n=160±24.56; Figure 1, G1-G3).

Among FB⁺ paravertebral non-catecholaminergic neurons, a discrete proportion was NPY-IR (11.10±1.71%), while smaller percentages of cells were nNOS-IR (2.53±1.34%) or LENK-IR (1.86±1.45%). Very few neurons exhibited immunoreactivity for ChAT (0.55±0.37%) or CGRP (0.34±0.18%). No non-catecholaminergic RCM-projecting neuron, in the studied ganglia, showed immunoreactivity for VIP or for SP alone.

Discussion

**TH-immunoreactivity**

On the basis of the presence of the catecholamine-synthetizing enzyme (TH), the majority of the autonomic paravertebral RCM-projecting neurons is catecholaminergic in character. These neurons are probably the major source of the high number of TH-IR fibers that we have previously found in the RCM (Gazza et al., 2005).

However, among the FB⁺ tested cells, there was a conspicuous population of neurons that did not express positivity to TH and that therefore, should be considered either cholinergic or non-catecholaminergic/non cholinergic in character.

Noradrenaline is excitatory in nature and causes contraction of smooth muscle cells (Owman and Stjernquist, 1988; Keast, 1999; Andersson, 2000). As noradrenergic fibers are usually found in the muscular coat or around blood vessels of genital organs (Kaleczyc et al., 1993; Czaja et al., 1996; Andersson and Stief, 1997), and of the RCM in particular (Gazza et al., 2005), it may be concluded that the TH-IR RCM-projecting neurons exert the same influence both on the smooth muscle cells of the muscle and its blood vessels.

**NPY-immunoreactivity**

NPY was the substance that showed the highest proportion of co-localization with TH. Our previous observations (Gazza et al., 2005) of high numbers of TH/NPY-IR fibers in the sow RCM are in accordance with the present finding. Moreover, high levels of NPY in noradrenergic neurons have already been found by Lundberg et al. (1983) and by Klimczuk (2004) in the male pig sympathetic paravertebral neurons.

In the present study, NPY-immunoreactivity was also found in a significant number of TH-negative (TH⁻) neurons innervating the porcine RCM. This is in accordance with the proportion of NPY-containing cells (5-10% of the gangliar neurons) found by Lakomy et al. (1994) in the thoraco-lumbar paravertebral ganglia of female piglets.

Our finding is not only in agreement with the large number of NPY-IR nerve fibres observed in the RCM (Gazza et al., 2005), but also, more generally, in different porcine female genital organs such as the uterus, oviduct and ovary (Häppölä et al., 1991).

Since NPY is a potent vasoconstrictor of many mammalian arteries (McLachlan and Llewellyn-Smith, 1986; Lindh et al., 1989; Lundberg et al., 1990), these NPY-positive neurons, whether containing TH or not, could play a role in blood flow regulation (Gibbins, 1991, Lundberg et al., 1983). Moreover, in the female, NPY also regulates the contractile activity of non-vascular smooth muscle cells of the uterus and oviduct, as well as the secretory function of the ovary (Markiewicz et al., 2003). These regulatory functions might be performed by inhibiting the release of acetylcholine (ACh) from cholinergic nerve endings by a presynaptic mechanism (Stjernquist et al., 1987; Lundberg et al., 1990), or by enhancing the contractile effects of noradrenaline directly affecting postsynaptic receptors (Ekblad et al., 1984).

**nNOS-immunoreactivity**

In the present study, nNOS showed a moderate degree of co-localization with TH within the porcine paravertebral RCM-projecting neurons. This type of immunoreactivity has not been observed either in the nerve fibres supplying the sow RCM (Gazza et al., 2005) or in the fibers and neurons innervating the porcine female reproductive organs (Majewski et al., 1995). Differently, the co-localization of nNOS with TH had earlier been observed in some paravertebral neurons projecting to the bulbospongiosus muscle of the male pig (Gazza et al., 2003) and, but only exceptionally, in a very small number of paravertebral neurons of the cat (Anderson et al., 1995), guinea-pig (Fischer et al., 1996), and man (Klimaszewski et al., 1996). Finally NOS has been shown to be co-localized with dopamine-beta-hydroxylase (DBH)
in the dopaminergic neurons of the bovine cranial cervical ganglion (Sheng et al., 1993).

We have detected nNOS-immunoreactivity also in a small number of TH neurons projecting to the RCM. This immunoreactivity has already been demonstrated in a small number of paravertebral neurons supplying the gilt genital organs (Majewski et al., 1995), and in non-noradrenergic cells (Anderson et al., 1995; Fischer et al., 1996), where nNOS is often co-localized with VIP or NPY.

In the porcine female genital organs, Majewski et al. (1995) found high numbers of nNOS-IR fibers only in the ovary and moderate numbers of nitrergic fibers in the oviduct, uterus and vagina. These results indicated that NO is particularly involved in the regulation of blood flow through the porcine reproductive tract. It may be postulated that NO also exerts its well known relaxing activity on the smooth muscle cells of the RCM, in addition to causing vasodilatation of its blood vessels.

On the other hand, in its co-existence with TH, might play a role of modulator. In fact, it seems seem to lead to an increase in TH activity in post-ganglionic neurons via both cyclic GMP-dependent and -independent mechanisms (Klimaschewski et al., 1996).

LENK-immunoreactivity

We found immunoreactivity for LENK, an enkephalin belonging to the opioid peptides family, in a fair number of catecholaminergic RCM-projecting cells of the PaG S1. The coexistence of LENK or other enkephalins with TH in pig paravertebral neurons has been observed only in some neurons projecting to the bulbospongiosus muscle (Gazza et al., 2003), but opioid peptides have been detected in noradrenergic nerve terminals supplying both the muscle layer and some arteries of the porcine oviduct (Czaja et al., 1996).

We also found LENK immunoreactivity in a small number of FB+/TH− neurons of the PaG S1. In the pig, the presence of opioid peptides has been noticed in neurons of the cervical (Häppölä et al., 1993), thoraco-lumbar (Lakomy et al., 1994) and sacral (Gazza et al., 2003; Botti et al., 2006) paravertebral ganglia, independently of the presence of TH. Moreover, LENK-immunoreactivity has been found in nerve processes in the interstitial connective tissue of the sow RCM (Gazza et al., 2005) and, in general, opioid peptides have been seen in fibres innervating the pig female genital organs (Czaja et al. 1996).

The LENK-containing neurons might have a regulatory role in neurotransmission in the RCM and its vessels. In fact, the coexistence of enkephalins with noradrenaline in sympathetic ganglia (Konishi et al., 1981) and in fibres innervating genital organs (De Potter et al., 1987) suggests that these peptides may presynaptically modulate sympath-
ic inputs at the neuro-effector junction (Czaja et al., 1996). The same coexistence, observed in several noradrenergic perivascular nerve fibers (Owman and Stjernquist, 1988), suggests that enkephalins might also have a vasodilator effect due to their inhibitory action on autonomic neurotransmission (Konishi et al., 1981; Kaleczyc et al., 1997; Kaleczyc, 1998).

**CGRP-immunoreactivity**

The present study proved the co-localization of CGRP with TH in a small number of RCM-projecting cells. Identically coding neurons have been already observed among the ones projecting to the pig bulbospongious muscle (Gazza et al., 2003) and in human paravertebral ganglia (Baffi et al., 1992). However, like SP, CGRP is a tachykinin commonly considered to be a marker of afferent pathways (Majewski et al., 1995; Kaleczyc et al., 1997; Czaja, 2000); therefore, its presence within paravertebral neurons is an unforeseen finding and its co-localization with TH even more so.

The detection of only two TH+/CGRP+ neurons is another unexpected finding because CGRP-immunoreactivity has already been described in pig (Häppölä et al., 1993; Lakomy et al., 1994; Gazza et al., 2003), cat (Lindh et al., 1989) and horse (Nasu et al., 2003) paravertebral ganglia. However, our data of a very small number of CGRP+ neurons are in accordance with the infrequent findings of CGRP-IR fibers in the interstitial connective tissue of the sow RCM (Gazza et al., 2005). Until now, the co-localization of TH and VIP has been described only in sacral paravertebral neurons projecting to the pig bulbospongious muscle (Gazza et al., 2003). However, VIP has already been found in association with DβH in some vasodilatory neurons of the porcine thoracic sympathetic chain ganglia (Hill and Elde, 1989; Majewski, 1999). The coexistence of TH and VIP is unusual for a peptide commonly thought to be a marker of the cholinergic pathway or a classical neurotransmitter of the inhibitory-non adrenergic/non cholinergic (NANC) subdivision of the autonomic nervous system (Itoh et al., 1995).

In general, VIP-immunoreactivity has been found, with distinct cranio-caudal differences, in some neurons of the pig cervical (Häppölä et al., 1993), thoraco-lumbar (Hill and Elde; 1989; Lakomy et al., 1994) and sacral paravertebral ganglia (Botti et al., 2006). VIP-ergic neurons play a role in blood flow regulation, because, like NO, VIP acts as vasodilator on genital organ blood vessels (Polak et al., 1981; Morris et al., 1985; Mayewski et al., 1995), and displays a physiological antagonism with NPY in the control of blood flow (Morris et al., 1985).

**ChAT-immunoreactivity**

The co-localization of cholinergic and catecholaminergic markers, detected in a small number of RCM projecting neurons has been observed only in paravertebral neurons projecting to the pig bulbospongious muscle (Gazza et al., 2003). On the other hand, ChAT and DβH have been found co-localized in the foetal pig superior cervical ganglion (Wang et al., 1995). It is known that, during foetal development, some neurons transiently express the noradrenergic phenotype but they subsequently lose their noradrenergic characteristics, becoming cholinergic cells (Schäfer et al., 1997; Keast, 1999; Masliukov and Timmermans, 2004). If so, we may suggest that, in the gilt sympathetic neurons observed herein, the development of the cholinergic marker might not have been paralleled by a complete disappearance of the various catecholamine markers.

A very scarce number of non-catecholaminergic neurons projecting to the RCM were ChAT-IR. This finding is in accordance with the fact that only single cholinergic fibers have been observed in the
interstitial connective tissue and in the muscular coat of the vessels of the pig RCM (Gazza et al., 2005) and of the male homologous retractor penis muscle (Majewski et al., 1999).

Only small groups of cholinergic neurons have also been observed among the paravertebral neurons projecting to the RPM in the male pig (Botti et al., 2006) and in the sympathetic chain ganglia (Alaranta et al., 1997) or bull (Alaranta et al., 2000). This is in accordance with the fact that the cholinergic paravertebral neurons target principally eccrine sweat glands or hindlimb blood vessels (Majewski, 1999). The small number of cholinergic structures found in the above-mentioned studies might also be due to the limited availability and sensitivity of a suitable marker for cholinergic neurons in the peripheral nervous system (Kaleczyc, 1998; Klimczuk et al., 2005), or to the fact that single cholinergic nerve cell bodies and small acetylcholinesterase-positive ganglia are localized within the muscle itself, rather than in other autonomic ganglia, as has been shown in species such as the goat (Sjöstrand et al., 1993) or bull (Alaranta et al., 1989). Finally, in many species, including the pig, CHAT- and VACHT (vesicular acetylcholine transporter)-immunoreactivity are present in a number of endothelial cells (Haberberger et al., 2000). Therefore, there would be a second source of ACh that could take part in some regulatory mechanisms.

The principal function of cholinergic innervation in the urogenital system might be the presynaptic inhibition of the adrenergic excitatory neurotransmission (Klinge and Sjöstrand, 1977; Sjöstrand et al., 1993), and this could not require a high number of cholinergic neurons and nerve fibers, that, at least as far as the control of the blood flow is concerned, could be helped by the endothelial cells releasing ACh.

Conclusions

The RCM is essentially an isolated bundle of smooth musculature that, differently from other smooth muscles associated with the genital organs, is not a layer of a tubular organ or a part of the fibromuscular stroma of a gland. For this reason, we can be sure that the neurons labelled by the retrograde tracer are the ones exclusively directed to the smooth myocytes of the RCM or of its vessels and to no other tissue component (e.g. glands) of a genital organ. The simplicity of the preparation and the reliability and reproducibility of the obtained data make the RCM a useful model for the study of the innervation of the smooth muscles associated with genital organs.

The present findings suggest that the neurons of the PaG S1, that mainly contributes to the sympathetic innervation of the sow RCM, is involved, first of all, in the maintaining of the muscle tonus of both non-vascular and vascular smooth myocytes. A minor proportion of ganglionic paravertebral cells may participate to the modulation (regulation) of neurotransmission or they may act as inhibitory neurons on the activity of non-vascular smooth myocytes, while neurons with only a vasodilator function seem to be very scarce.

Finally, our findings seem to confirm the presence of a previously nearly unknown small population of CGRP- and SP-IR catecholaminergic sympathetic neurons, and indicate the existence of a small proportion of tachykinergic/non-catecholaminergic sympathetic neurons.

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