INTRODUCTION

Lower urinary tract is composed of urinary bladder and urethra. Both organs have to fulfill very specific functions during miction: while urinary bladder is a kind of a storage reservoir, urethra acts as a "biological valve" being able to assure the continence. As both organs must cooperate in a very fine balance, the crucial role of the neural control of their functions is secured by the afferent arch of the micturition reflex. Although domestic pig has been regarded as a more suitable animal model for studying human lower urinary tract innervation than rodents or carnivora (1), as of now bladder afferent neurons have been identified only in rat (Th13-L2 and L6-S1 DRGs), guinea pig and cat (Th11-L2 and S2-S4 DRGs) (2-4). Although immunohistochemical (IHC) studies of these neurons showed presence of various neuropeptides, such as SP, CGRP, vasoactive intestinal polypeptide (VIP) and leucine-enkephalin (ENK) (5, 6), there is still paucity of data concerning the chemical coding of porcine bladder-projecting DRG neurons. Therefore, we have undertaken the present study, combining both the retrograde neuron-tracing and standard IHC techniques, to provide detailed description of the spatial distribution and neurochemical coding patterns of neurons supplying the porcine urinary bladder.

MATERIALS AND METHODS

Investigations were performed on six immature Great Polish White female pigs (aged 8-12 weeks, 15-20 kg b.w.), kept under standard laboratory conditions with free access to water. Surgical procedures were applied in agreement with the guidelines of Local Ethical Committee under deep barbiturate anaesthesia. All animals were pretreated with atropine (Polfa, Poland; 0.04 mg/kg b.w., s.c.) and propionylpromasine (Janssen Pharmaceutica, Belgium; 0.5 mg/kg b.w., i.m.) thirty minutes before the main anesthetic, sodium pentothal (Sandoz, PL, 0.5 g.

Pig has been used recently as an animal model for studying diseases of human urinary tract, however, the sensory innervations of urinary bladder in this species has not been yet described. Objective: The present study was aimed at neurochemical characterization of sensory neurons of dorsal root ganglia (DRGs) supplying porcine urinary bladder.

Methods: Retrograde tracer Fast Blue (FB) was injected into the right half of the urinary bladder wall of six juvenile female pigs. Three weeks later ipsi- and contralateral DRGs of interest were harvested from all animals and a neurochemical characterization of retrogradely-labeled neurons was performed using routine single-immunofluorescence labeling technique on 10 µm-thick cryostat sections. Results: 85% of spinal sensory neurons supplying porcine urinary bladder was located in ipsilateral sacral S1-S4 ganglia and in first coccygeal ganglion (Cq1), whereas rest of FB-positive (FB+) nerve cells were found in lumbar L1-L4 DRGs. FB+ neurons belonged mostly to the medium-sized (54%) and small-sized afferent perikarya (45%). Bladder sensory neurons contained substance P (SP), calcitonin gene-related peptide (CGRP), pituitary adenylate cyclase-activating peptide (PACAP), galanin (GAL), neuronal nitric oxide synthase (nNOS), somatostatin (SOM) and/or calbindin-28k (CB), and these neurons constituted 45%, 36%, 26%, 6%, 6%, 4% and 3% of all retrogradely traced DRGs perikarya, respectively. Distinct differences in the number of traced cells and their neuropeptide content were observed between the lumbar and sacral/coccygeal division of bladder-projecting DRG neurons. Thus, FB+ neurons expressing CGRP, GAL, nNOS or SOM were more numerous in lumbar DRGs (44%, 9%, 9% and 6%, respectively), than in sacral/coccygeal ganglia (23%, 2%, 1.5% and 0.3%, respectively). On the other hand, more FB+ cells expressed PACAP in sacral (31%) than in lumbar DRGs (23%). However, fractions of SP-IR or CB-IR bladder sensory neurons were similar in lumbar and sacral/coccygeal DRGs. Conclusions: This novel description of both spatial and neurochemical organization pattern of porcine urinary bladder sensory innervation constitutes a basis for further functional studies aimed at unraveling neurogenic mechanisms of urinary bladder diseases.

Key words: pig, urinary bladder, immunohistochemistry, dorsal root ganglia, neuropeptides
per animal) was given intravenously in a slow, fractionated infusion. After a mid-line laparotomy, the urinary bladder was gently exposed and a total volume of 40 µl 5% aqueous solution of the fluorescent retrograde tracer FB (Dr K. Illing KG & Co GmbH, Germany) was injected into right side of urinary bladder wall. All animals were killed after three weeks (the optimal survival period to allow the tracer to travel to dorsal root ganglia) by an overdose of sodium pentobarbital and, after the cessation of breathing, perfused transcardially with freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Bilateral spinal ganglia, together with the spinal cord segments Th0 to Cq4, were collected from all six animals studied. Tissue samples were postfixed in the same fixative for 10 min., washed several times in 0.1 M phosphate buffer and stored in 18% buffered sucrose at 4°C until sectioning. Serial cryostat sections 10 µm thick from all DRGs were examined using a fluorescence Olympus BX51 microscope equipped with an appropriate filter set. Only FB+ neurons with clearly visible nuclei were counted in every fourth section. The number of FB+ neurons found in all DRGs from particular animal as well as the relative frequency of perikarya belonging to the particular neuronal classes were averaged and presented as mean ± SEM.

RESULTS

FB-positive primary sensory neurons supplying urinary bladder were found in bilateral DRGs from lumbar segments L1–L5 and from sacral-coccygeal segments S3–Cq1 of spinal cord three weeks after administration of FB into right half of the urinary bladder wall. The number of FB+ neurons in six studied ipsi- and contralateral DRGs ranged per animal from 350 to 520 retrogradely labeled perikarya (430.5±23.9).

A distinct organization of afferent neurons projecting to the urinary bladder was observed in terms of both ipsi- and contralateral distribution pattern. Approximately 90% of all FB-positive spinal sensory neurons (89.2±1.2%) were located in the ipsilateral ganglia, while the rest (10.8±0.9%) of retrograde-labeled bladder sensory neurons were observed in contralateral DRGs.

The vast majority (85%) of ipsilateral sensory neurons supplying porcine urinary bladder was located in sacral S3–S4 ganglia and in first coccygeal ganglion Cq1 (16.8±0.6%, 50.2±4.4% and 18.2±0.7%, respectively). The remaining 15% of FB+ nerve cells were found in lumbar ganglia from L1 to L5 (6.6±0.6%, 4.9±0.2%, 2.3±0.2% and 1.0%, respectively).

FB-positive neurons belonged to three size classes of afferent perikarya: medium-sized cells prevailed over the small-sized bladder sensory neurons (54.2±4.4% vs. 45.4±3.9%, respectively), while the FB+ large sensory neurons were only occasionally found in DRGs studied (0.4±0.2%). Moreover, differences in the proportions of neurons which belonged to particular size-classes were observed between lumbar and sacral/coccygeal DRGs. In lumbar DRGs most retrogradely traced neurons were medium-(62.9±4.4%) and small-sized (37.1±4.5%), while large sensory neurons were not found. In sacral and coccygeal DRGs, FB+ cells consisted mainly of small-sized neurons (59.1±1.3%) while medium-sized perikarya were less numerous (39.1±1.9%).
Sporadically, these ganglia contained also single retrogradely traced cells of large diameter (1.8±0.2%). The presence of various neurotransmitters or their synthesizing enzymes (nNOS) in retrogradely labelled FB+ bladder sensory neurons was demonstrated by IHC. The most numerous bladder afferent neurons were SP-IR (Fig. 1b), constituting 45.2±4.4% of all FB-positive cells. CGRP was found in 36.1±4.7% of all retrograde traced DRG neurons (Fig. 2b); this neuropeptide was observed in perikarya of all size-classes. One fourth (26.1±3.3%) of bladder afferent neurons contained PACAP and these cells were mainly small in size (Fig. 3b). Only few FB+-small neurons in DRGs expressed GAL-IR (Fig. 4b, 6.5±2.2%); similarly, only few nNOS- or SOM-IR bladder afferent cells were observed in DRGs studied (5.8±2.5% and 3.7±2.8%, respectively). Neuronal NOS was mainly found in medium-sized DRG neurons (Fig. 5b), while all SOM-IR FB-positive cells had small diameter (Fig. 6b). Single, medium size DRG neurons showed CB-IR (2.8±1.4%, Fig. 7b). It should be stressed that about one third (35.9±2.8%) of all FB+ cells did not exhibited immunoreactivity against any biological active substance investigated.

There were significant differences in the relative frequencies of neurons containing particular neurotransmitters between lumbar and sacral/coccygeal DRGs. Although retrogradely labeled (FB+) neurons were less numerous in lumbar DRGs, more FB+ cells immunolabeled to CGRP, GAL, nNOS and SOM were observed in these ganglia (44.4±4.3%, 9.3±3.8%, 8.6±3.8% and 6.1±4.6%, respectively), when compared to sacral/coccygeal ganglia (23.5±5.9%, 2.4±0.5%, 1.5±0.5% and 0.3±0.3%, respectively). On the other hand, more retrogradely labeled cells in S1 and S2 DRGs contained PACAP (31.2±4.0%) as compared to FB+-lumbar neurons (22.8±4.6%). There were no statistically significant differences in the number of bladder sensory neurons immunolabeled for SP or CB between lumbar and sacral/coccygeal DRGs studied.

**DISCUSSION**

This is the first report that demonstrates the detailed distribution pattern of porcine urinary bladder-projecting DRG neurons, as well as reveals their neurotransmitter content. As of now, the only available data concerning the distribution of bladder-projecting DRG neurons came from studies performed in rat, guinea pig and cat. Some authors showed that in the rat urinary bladder-projecting DRG neurons were found exclusively in DRGs L5 and S1 (9), however, in another study, rat bladder afferent neurons were identified by retrograde axonal transport in DRGs L1, L2, L5 and S1 (9-11). On the other hand, Calsen-Cencic and Mense (12) showed that rat urinary bladder receives “dual” afferent innervation originating from thoracolumbar (Th1-L3) and lumbarosacral (L5-S5) DRGs. These conflicting data obtained in the same species probably may be attributed to various application method and/or amount of the tracer injected. In guinea pig (3) the majority of bladder afferent neurons was located in DRGs L5-S5 and only a few in DRGs L1-L3. Thus, our present results and data of other authors (3, 9-11) suggest that in all species studied so far bladder afferent neurons are present in two groups of DRGs: a minority within the DRGs of thoracolumbar neurones and the majority in the caudal lumbar and sacral/coccygeal DRGs.

In rat fraction of SP-IR bladder-projecting neurons ranged from a very small number (3% as reported by Vizzard (13) and Calsen-Cencic and Mense (12)) to about one third (36%) as reported by Wang et al (1). This latter report is in line with the data obtained in the present study in pig. The presence of SP in afferent neurons (14, 15) may be involved in mediating urinary bladder hyperreflexia (16, 17) and inflammation, as the release of SP in the bladder wall is known to trigger inflammatory responses including plasma extravasation and/or vasodilation (18). As central branches of SP-IR, bladder-projecting DRG neurons project to the dorsal part of the sacral parasympathetic nucleus (6, 13) and since intrathecally applied SP facilitates normal micturition, it was suggested that this neuropeptide may be involved as an excitatory neurotransmitter in several types of bladder reflexes in rat (19). Furthermore, in this species SP was shown to facilitate the impulse activity of sympathetic preganglionic neurons in the intermediolateral nucleus in thoracolumbar spinal cord (20), which form the origin of the sympathetic (inhibitory) innervation of the bladder. Thus, it is possible that also in pig SP may be involved in the regulation of urinary bladder functions at different levels.

In contrast to other species, in which expression pattern of CGRP in DRGs neurons ranged from 50% (13, 21) through 60% (12, 22) up to 70% (1) of all bladder-projecting DRG cells, much smaller fraction (36%) of CGRP-IR sensory neurons supplying urinary bladder was observed by us in the pig. Similarly to rat (1, 22), porcine bladder sensory neurons belonged to all three size-classes of afferent neurons. Previous studies suggest that CGRP acts synergistically with SP in spinal cord (23). Such synergism may result from the CGRP-mediated inhibition of an endopeptidase which degrades SP (24), thus elevating the local concentration of SP at the site of release, or by an enhancement of the SP-release from primary afferent fibers in the spinal cord by CGRP as suggested by Oku et al. (25). In this context our results indicate that CGRP, which by itself has no excitatory effect on the vesico-vesical reflex pathway (26), may facilitate the SP-evoked chemonociceptive reflex.

In rat approximately 40-45% of bladder afferent cells in the L5-S5 DRG exhibited PACAP immunoreactivity (1, 11), whereas in porcine DRGs we observed smaller proportion of PACAP-IR neurons in retrogradely-traced perikarya. Although the exact physiological role of PACAP in lower urinary tract is still unclear, it was suggested that PACAP may be involved in the facilitation of spontaneous bladder contractions (27).

Our observation that a small population of porcine FB+ DRG neurons expressed GAL-IR corresponds well (with one exception, 28) with the results obtained in rat (1, 12). GAL was shown to influence the activity of vesical smooth muscles of rat urinary bladder and modulate neural transmission both in autonomic ganglia (29) and at neuromuscular junctions where GAL suppressed the cholinergic component of the response to electric field stimulation (30). Thus, an inhibitory action of GAL on neurotransmitter release has been suggested in smooth muscle tissues and this may also pertain to urinary bladder (30). Moreover, some anti-inflammatory properties of GAL were suggested, since it was shown to presynaptically inhibit the release of SP and CGRP from capsaicin-sensitive primary afferents (31). When administrated intrathecally in higher doses GAL also blocked the facilitatory effects of SP and CGRP on the excitability of the nociceptive flexor reflex in rat (32, 33).

Similarly to data presented in rat (12) we also found that neuronal NOS-IR retrogradely labelled neurons constituted a relatively small population of mostly medium-sized perikarya. However, in guinea pig (3) the proportion of NOS-IR bladder afferent neurons in in L5-S5 DRGs was twice numerous than in rat (12) or pig (present data). In contrast, Vizzard (10) showed that in rat FG-labeled bladder afferent neurons were entirely NOS-immunonegative. Being a small, reactive and gaseous molecule which is able to easily pass through neuronal membranes, NO may act as a "retrograde transmitter" in the sensory pathways, playing a pivotal role in nociceptive processing in multisynaptic local circuits of spinal cord (34).
since exogenously applied NO inhibited Ca\(^{2+}\) channels in dissociated lumbosacral dorsal ganglion neurons innervating the urinary bladder (35). Furthermore NO may be involved in the facilitation of the micturition reflex by nociceptive afferents at the spinal cord level (10). Functional experiments showed that NOS-IR fibers inhibited trigonal, bladder neck and urethral smooth muscle tone (36, 37).

Only very few SOM-IR sensory cells supplying rat urinary bladder were found in rat DRGs and all of them were small in size (1). Similar results were observed in our study. There is a lack of data with regard to a putative physiological relevance of this subclass of urinary bladder afferent neurons.

In conclusion, the present study provides for the first time a detailed description of both the spatial and neurochemical organization pattern of porcine urinary bladder sensory innervation. The results may be helpful in establishing a morphological basis for further functional experiments in a porcine animal model focusing on the mechanisms of neurogenic diseases of the urinary bladder.

Conflict of interests: None declared.

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