Abstract  The plant lectin, IB4, binds to primary afferent neurons of dorsal root and trigeminal ganglia, where it is selective for nociceptive neurons. In the enteric nervous system of the guinea-pig IB4 labels intrinsic primary afferent neurons, which are believed to have roles as nociceptors. Here we investigate whether IB4 binding is also a marker of intrinsic primary afferent neurons in the mouse. Neurons that bound IB4 were common in the enteric plexuses of the small intestine and colon. Labeled neurons were rare in the stomach, and absent from the esophagus and gall-bladder. Binding was to the cell surface, initial parts of axons and to clumps in the cytoplasm. Similar binding occurred on small and medium sized neurons of dorsal root, nodose and trigeminal ganglia. In the enteric nervous system, IB4 revealed large round or oval (type II) neurons, type I neurons with prominent laminar dendrites and small neurons of myenteric ganglia. The type II neurons were immunoreactive for calretinin, and some type I neurons were immunoreactive for nitric oxide synthase. Most neurons in the submucosal ganglia bound IB4, and some of these were vasoactive intestinal peptide immunoreactive. Thus IB4 binds to specific subgroups of enteric neurons in the mouse. These include intrinsic primary afferent neurons, but other neurons, including secretomotor neurons, are labeled. The results suggest that IB4 is not a specific label for enteric nociceptive neurons.

Keywords  Enteric nervous system · Lectin binding · Primary afferent neurons · Nociceptors

Introduction

Recent studies have suggested that IB4, a plant lectin from Bandiera simplificifolia, binds selectively to nociceptive neurons in the trigeminal and dorsal root ganglia (Stucky and Lewin 1999; Gerke and Plenderleith 2001). There is evidence that the enteric nervous system of the gastrointestinal tract also contains nociceptive neurons (Furness 2006). Noxious stimuli encountered by the intestine include the presence in the lumen of plant toxins, harmful bacteria, toxic bacterial products, and certain viruses, such as rotavirus. The neurons within the intestine react to the presence of the noxious agents by initiating motility reflexes to expel the toxins and secretory reflexes to dilute them (Mathias et al. 1982; Cowles and Sarna 1990; Lundgren 2002). Analysis of the intrinsic reflex pathways has identified intrinsic primary afferent neurons (IPANs) as the nociceptive neurons within the gut wall (Furness 2006). Whether the IPANs bind IB4 has only been investigated in the guinea-pig (Hind et al. 2005), and relies on the morphological identification of intrinsic primary afferent neurons (IPANs), which are large round or oval type II neurons in that species (Furness et al. 2004a). The IPANs detect changes in the physiological state of the intestine and, indirectly, they detect chemicals within...
the luminal contents. IPANs share a number of morphological, neurochemical and electrophysiological similarities with small diameter C-fiber primary afferent neurons with cell bodies in dorsal root and cranial sensory ganglia, that have been identified to be nociceptors. Both types of neuron have unmyelinated axons and, similar to IPANs, many small diameter neurons of dorsal root ganglia (DRG) have broad action potentials with a hump on the falling phase (Djouhri et al. 1998). As is the case for IPANs, the broad action potential is generated through a mixture of tetrodotoxin-sensitive Na+, tetrodotoxin resistant Na+, and Ca2+ currents (Yoshida et al. 1978). In both types of neuron, Ca2+ that enters during the action potential contributes to the generation of a Ca2+-dependent K+ current that underlies an after-hyperpolarizing potential (Hirst et al. 1985; Djouhri et al. 1998). IB4 binds specifically to a-D-galactose end-groups of glycoconjugates (Lahtinen 1987), implying that such carbohydrates are expressed at the surfaces of IPANs (at least in guinea-pigs), DRG and trigeminal ganglion neurons.

Type II neurons have only recently been investigated in the enteric nervous system of the mouse (Furness et al. 2004b; Neylon et al. 2004; Nurgali et al. 2004). These neurons in the mouse closely parallel those in the guinea-pig in the projections of their processes, in their size and shapes, aspects of their neurochemistry and in their electrophysiological characteristics. Nevertheless, parallels between neuron morphology, electrophysiological characteristics, and indeed in the organization of the enteric plexuses, are imprecise across species (Brehmer et al. 1999; Timmermans et al. 2001; Furness 2006). Because of the increasing use of mouse models, especially genetically manipulated animals, in gastrointestinal research, and the need to understand whether the observations in the guinea-pig are applicable to other species, we decided to investigate whether the close correlation between type II morphology and IB4 binding also occurs in the mouse enteric nervous system.

Materials and methods

Mice of either sex (20–30 g) were killed by cervical dislocation and severing the spinal cord or were perfused as described below. All procedures conformed to National Health and Medical Research Council of Australia guidelines and were approved by the University of Melbourne Animal Experimentation Ethics Committee. All observations were repeated on specimens from at least three mice. For tissue that was to be examined in wholemount, we removed segments of esophagus, gastric fundus, body and antrum, ileum, distal colon and gallbladder from 15 mice and fixed them for immunohistochemistry. The segments of gastrointestinal tract were cleaned of contents, opened along the mesenteric attachment and pinned to balsa wood sheets for fixation in 2% formaldehyde plus 0.2% picric acid in 0.1 M sodium phosphate buffer, pH 7.2, at 4°C. Fixation was continued overnight at 4°C. Preparations were cleared of fixative by 3 × 10 min washes in dimethyl sulphoxide (DMSO) followed by 3 × 10 min washes in phosphate-buffered saline (PBS: 0.15 M NaCl in 0.01 M sodium phosphate buffer, pH 7.2). Fixed tissue was stored at 4°C in PBS containing sodium azide (0.1%).

The mucosa, submucosa and circular muscle were removed from fixed tissue, and wholemounts consisting of the myenteric plexus adhering to the longitudinal muscle were prepared. Wholemount preparations were incubated in 10% normal horse serum (NHS) plus 1% triton X-100 in PBS for 30 min prior to exposure to fluorescein isothiocyanate (FITC) labeled IB4 (FL-1201, 5µg/ml, Vector laboratories, Burlingame, CA, USA) or labeled IB4 plus antibodies to markers of different classes of enteric neurons: rabbit anti-calretinin antibodies (SWant, Bellinzona, Switzerland), 1:1000; rabbit anti- neuronal nitric oxide synthase (NOS) (Anderson et al. 1995), 1:1000; or rabbit anti-vasoactive intestinal peptide (VIP) (Furness et al. 1981), 1:200. Wholemounts were incubated with primary antibodies overnight at 4°C. The wholemounts were then washed (3 × 10 min) in PBS before incubation with secondary antibodies (donkey anti-rabbit IgG, Alexa 594 labeled, Molecular Probes, Eugene, OR, USA) for 1 h at room temperature. Preparations were given 3 subsequent 10 min washes in PBS and then mounted on glass slides using a commercially available mounting medium (Dako Corporation, Carpinteria, CA, USA).

Tissue to be examined in sections was taken from 3 perfused animals. Mice were anaesthetized with sodium pentobarbital (120 mg/kg), given intra-peritoneally, and were perfused through the heart, with the same fixative that was used for immersion fixation. Dorsal root, trigeminal and nodose ganglia were removed after perfusion and further fixed overnight at 4°C. Tissue was cleared in DMSO as described above and placed in PBS-sucrose-azide (PBS containing 0.1% sodium azide and 30% sucrose as a cryoprotectant) and stored at 4°C overnight. The following day, ganglia were transferred to a mixture of PBS-sucrose-azide and OCT compound (Tissue Tek, Elkhart, IN, USA) in a ratio of 1:1 for a further 24 h before being embedded in