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Cellular distribution of vanilloid VR1 receptor immunoreactivity in the guinea-pig myenteric plexus

Sharon Anavi-Goffer, Angela A. Coutts*

Department of Biomedical Science, Institute of Medical Sciences, University of Aberdeen, Aberdeen, Scotland AB25 2ZD, UK

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Abstract

Recent investigations suggest that vanilloid receptor-1 (VR1) immunoreactivity occurs in the intestine. We have determined and quantified this immunoreactivity in the myenteric plexus with respect to cholinergic and neurofilament protein-positive neurones. Guinea-pig and rat preparations were dual-labelled with specific antibodies raised in rabbit or goat against vanilloid receptor-1 and against other neurochemical markers. In the rat ileum, both vanilloid receptor antibodies were co-distributed, whereas in the guinea-pig ileum and colon, tertiary fibres were also detected with the goat antibody. In the guinea-pig, all vanilloid receptor-1-immunoreactive cell bodies were choline acetyltransferase-immunopositive (100%) and showed some immunoreactivity to neurofilament proteins (NFP-200 kDa (79%) or triplet (10.8%)) or calretinin. Immunoreactive fibres in the secondary plexus co-localised with calcitonin gene-related peptide (CGRP) and with substance P, calretinin and synapsin I in the tertiary plexus. Subpopulations of cholinergic neurones including sensory, interneuronal and secretory neurones express vanilloid receptor-1. Co-localisation with substance P and calretinin in fibres suggests that vanilloid receptor-1 may be expressed by excitatory motor neurones. The association of vanilloid receptors with calcitonin gene-related peptide and synaptic protein in fibres implies a role for vanilloid receptors in neurotransmitter/neuropeptide release. Although it is likely that at least some of the vanilloid receptor-bearing fibres originate in immunopositive myenteric soma, the origin of all these fibres cannot be identified in the present study.

Keywords: Immunohistochemistry; Myenteric plexus-longitudinal muscle; Choline acetyltransferase; Capsaicin receptor; Confocal microscopy

1. Introduction

The responses of nociceptors to the exogenous vanilloid ligand, capsaicin, and to the endogenous cannabinoid ligand, anandamide, are at least partly mediated by vanilloid receptor-1 (VR1) (Caterina et al., 1997; Zygmunt et al., 1999). In the rat and guinea-pig intestine, the effects of capsaicin have been widely investigated (Allescher et al., 1992; Maggi et al., 1987). At low concentrations, capsaicin can trigger the release of neuropeptides and neurotransmitters from the peripheral terminals of neurones in the gut (Maggi, 1995). In the guinea-pig isolated ileum, capsaicin activates cholinergic neurones, which release acetylcholine (Barthó and Szolcsányi, 1978; Barthó and Vizi, 1985). It has been suggested that other transmitters such as calcitonin gene-related peptide (CGRP) (Barthó et al., 1991), possibly ATP (Barthó et al., 2000) and some unidentified transmitters are also released by capsaicin (Barthó et al., 1999). However, the contractile response to capsaicin is thought to be mainly due to the release of neurogenic substance P (Barthó et al., 1982). Providing there is a background of motor activity, capsaicin can produce relaxation due to the release of CGRP (Barthó et al., 1991). In the isolated guinea-pig ileum, anandamide increases the basal level of acetylcholine release, an action inhibited by the vanilloid VR1 receptor antagonist, capsazepine (Mang et al., 2001). When administered in vivo, capsaicin inhibits guinea-pig distal colonic motility by activation of postganglionic sympathetic nerves (Maggie et al., 1987). However, in vitro analysis of the responses to capsaicin reveals a variety of direct and indirect mechanisms. It mediates excitation of the cholinergic innervation of the smooth muscle and inhibits nonadrenergic, noncholinergic (NANC)-mediated spontaneous activity. In the guinea-pig isolated intestinal preparations, tetrodotoxin-resistant NANC responses have been shown to result from tachykinin-induced activation of neuronal tachykinin NK_1

* Corresponding author. Tel.: +44-1224-555739; fax: +44-1224-555719.
E-mail address: a.a.coutts@abdn.ac.uk (A.A. Coutts).

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receptors involved in the motor response to stimulation of capsaicin-sensitive primary afferent nerves (Venkova et al., 2002). Selective antagonism of capsaicin-induced activation of tachykinin NK_1 receptors can be achieved both in the spinal cord and at peripheral terminals implying an action on primary afferent fibres. In addition, it has been suggested that high concentrations of capsaicin inhibit intestinal smooth muscle directly (Maggi et al., 1987). These findings suggest putative functionally active vanilloid receptors on neuronal and possibly muscle elements in the small and large intestine; however, not all of these actions may be mediated by vanilloid type-1 receptors.

Recently, we have demonstrated that in the rat and guinea-pig ileum and colon myenteric preparations, vanilloid VR1 receptor immunoreactivity is located in neurones and fibres (Anavi-Goffer et al., 2002) where only a proportion of vanilloid VR1 receptor-positive neurones was immunoreactive for calbindin, a marker for intrinsic afferent neurones. In addition, vanilloid VR1 receptor labelling has been reported on myenteric fibres and variety of cells in cryostat sections and cultured neurones of rat and pig ileum (Akiba et al., 2001; Kulkarni-Narla and Brown, 2001). Taken together, these results imply that vanilloid VR1 receptors are expressed in more than one class of neurones in the myenteric plexus. In the present work, we have compared the distribution of vanilloid VR1 receptor immunoreactivity from two different antibodies raised in different species against the C terminus of the receptor. Double-stained preparations with selective markers for choline acetyltransferase, calretinin or neurofilament proteins identified subpopulations of neurones. The use of whole-mount preparations enabled us to investigate the co-localisation of markers on individual fibres and to quantify the proportion of vanilloid receptor-positive cells relative to markers for cholinergic neurones and neurofilament-expressing neurones. As capsaicin elicits the release of neurotransmitter and modulator peptides in the gut, we have also performed double labelling with selective antibodies for synaptic vesicular protein, CGRP and substance P.

2. Materials and methods

2.1. Tissue preparations

Segments of ileum and colon were isolated from male Dunkin–Hartley guinea-pigs (329–1115 g) killed by cervical dislocation and ileum from female Sprague–Dawley rats (140–175 g) that were anaesthetised with halothane inhalation and decapitated. These protocols conform to the European Community guidelines for the use of experimental animals, hence steps were taken to minimise trauma to animals and the number of animals used. Intestinal segments were placed in a warm (37 °C) Krebs solution and myenteric plexus-longitudinal muscle preparations were dissected as described previously (Anavi-Goffer et al., 2002). For fixation, tissues were pinned flat on a Sylgard-coated plate. The effects of different fixatives were examined in order to optimise labelling with vanilloid VR1 receptor antibodies. Tissues were fixed with either: (a) 4% paraformaldehyde (made in glucose-free HEPES-buffered saline (HBS)) for 10 min at 4 °C; (b) methanol for 30 min at −20 °C; (c) modified Zamboni's solution (2% paraformaldehyde and 0.2% picric acid in glucose-free HBS) overnight at room temperature. The picric acid solution was cleared with dimethyl sulphoxide then washed with buffer. Pieces of whole-mount tissue, cleared from circular muscle, were permeabilised and blocked with 1% Triton X-100 in 10% nonfat dried milk for 30 min at room temperature.

2.2. Immunohistochemical procedures

Vanilloid VR1 receptor immunoreactivity was detected with either rabbit or goat anti-vanilloid VR1 receptor polyclonal antibodies (Table 1) generated against a peptide of the predicted carboxyl terminus of rat vanilloid VR1 receptor origin (KPEDAEVFKDSMVPGEK). Permeabilised tissues were incubated overnight at 4 °C in 2% normal goat or donkey serum, 0.2% nonfat dried milk containing a mixture of primary antibodies (Table 1) in the appropriate concentrations. In each experiment, at least one of the primary antibodies in the mixture was vanilloid VR1 receptor antiserum. After washing, the tissues were incubated in a mixture containing the corresponding secondary antibodies (Table 2) for 1.5 h at room temperature. Excess secondary antibodies were removed with glucose-free HBS and tissues were mounted in Vectashield H-1000 (Vector Laboratories, Burlingame, CA, USA). Both vanilloid VR1 receptor antibodies were localised simultaneously in tissues fixed in methanol and then labelled with a mixture of Cy^3 donkey anti-rabbit and Alexa 488 donkey anti-goat secondary antibodies. In experiments that localised rabbit, anti-VR1 receptor antibody with choline acetyltransferase, neurofilament protein 200 (NFP-200), neurofilament triplet (NFT),

Table 1
Suppliers and dilutions of primary antibodies

Antibody	Host	Dilution	Supplier
Vanilloid VR1 receptor	rabbit	1:2500	Dr. D. Julius, San Francisco, CA (Tominaga et al., 1998)
Vanilloid VR1 receptor	goat	1:500, 1:1000	Santa Cruz Biotechnology
Choline acetyltransferase	mouse	1:250	Chemicon
Calretinin	mouse	1:100	Chemicon
NFP-200	mouse	1:500	Sigma
NFT	mouse	1:500	Dr. Vitadello, Padova, Italy (Costa et al., 1996)
Synapsin I	mouse	1:250	Chemicon
CGRP	mouse	1:100	RBI
Substance P	mouse	1:100	Immundiagnostik

Santa Cruz Biotechnology, Santa Cruz, CA, USA; Sigma–Aldrich, Dorset, UK; Chemicon International, Temecula, CA, USA; RBI, Natick, MA, USA; Immundiagnostik, Bensheim, Germany.

Table 2
Suppliers and dilutions of secondary antibodies

Antibody	Dilution	Supplier
Cy^3 donkey anti-rabbit	1:500	Jackson
Alexa 488 donkey anti-goat	1:1000	Molecular probes
Alexa 488 goat anti-mouse	1:1000	Molecular probes
Cy^5 donkey anti-mouse	1:250	Jackson

Jackson Immunoresearch Laboratories, West Grove, PA, USA; Molecular Probes-Europe, Leiden, The Netherlands.

calretinin or synapsin I monoclonal antibodies, a sequential procedure was used. Firstly, methanol-fixed tissues were labelled with a combination of the vanilloid VR1 antibody and the monoclonal antibody. After washing, the tissues were refixed in modified Zamboni's solution containing 0.2% picric acid and washed before labelling with a mixture of Cy^3 donkey anti-rabbit and Alexa 488 goat anti-mouse secondary antibodies. Synapsin I was also localised with goat anti-VR1 receptor in tissues that were fixed in methanol and visualised with a mixture of Alexa 488 donkey anti-goat and Cy^5 donkey anti-mouse antibodies. The fixation procedures that were used for each antibody combination are given in Table 3. Calretinin, CGRP and substance P monoclonal antibodies did not label well after methanol fixation, therefore, labelling with these antibodies in combination with anti-VR1 receptor antibodies was performed in tissues fixed in 4% paraformaldehyde or modified Zamboni's solution. These combinations of antibodies were visualised with a mixture of a Cy^3 donkey anti-rabbit and Alexa 488 goat anti-mouse or Alexa 488 donkey anti-goat and Cy^5 donkey anti-mouse antibodies.

2.3. Controls

Control experiments included the omission of primary antibodies and incubation with inappropriate secondary antibodies against vanilloid VR1 receptor antibodies; no labelling was observed. Rat and guinea-pig preparations were pre-absorbed with 100 μg/ml of vanilloid VR1 carboxyl terminus blocking peptide (Novartis or Santa Cruz Biotechnology) for 3 h at room temperature before labelling with either or both antibodies.

2.4. Image acquisition and processing

Images were taken with a Bio-Rad 1024 laser scanning confocal imaging system attached to a Nikon Diaphot 200 microscope. Cy^3 antibody was excited at 568 nm, Alexa 488 at 488 nm and Cy^5 at 647 nm, and fluorescent emission was detected at 585, 522 and 680 nm, respectively. Z-series of 3- to 5-μm-thick sections that were Kalman-averaged for four individual scans were obtained sequentially. Parent images were merged with LaserSharp software.

2.5. Quantification of co-labelled cells

The number of vanilloid VR1 receptor, choline acetyltransferase, NFP or NFT immunopositive cells was counted from the merged images from the guinea-pig ileum. The proportion of co-labelled cells relative to the total number of vanilloid VR1 receptor-positive cells or the co-label marker was calculated from a minimum of 367 vanilloid VR1 receptor-positive cells, labelled with rabbit anti-VR1 receptor antibody. Data are expressed as mean ± S.E.M. For statistical analysis, the value of n is the number of animals. Comparisons were made by Student's unpaired t-test, GraphPad Prism. P values <0.05 were considered to be significant. Cells co-labelled with other markers were not quantified since the number co-localised was too small for the proportion to be quantified with sufficient accuracy.

Table 3
Fixation procedures for each antibody and corresponding images

Primary antibody	Fixation procedure	Labels	Figure
Rabbit anti-VR1	methanol	cells and fibres in guinea-pig; fibres in rat	Figs. 1B–I and 3D–F
	4% *p*-formaldehyde	incomplete label of cells and fibres	Fig. 3G
	Zamboni's[a]	cells	Fig. 3H
	sequential	cells and fibres in guinea-pig and rat	Figs 1A, 2A–H and 3A,C
Goat anti-VR1	methanol	fibres in rat plus tertiary fibres in guinea-pig	Figs. 1B,D,F,G,H and 4A,C
	4% *p*-formaldehyde	tertiary fibres and cell nuclei in guinea-pig	Fig. 4J,L
	Zamboni's	tertiary fibres in guinea-pig	Fig. 4D,F,G,I
Choline acetyltransferase	sequential	cells in guinea-pig ganglia	Fig. 2B
NFP-200	sequential	cells and fibres in guinea-pig ganglia	Fig. 2F
NFT	sequential	as above	Fig. 2H
Substance P	methanol	fibres in guinea-pig	Fig. 3E,F
	Zamboni's	tertiary fibres and ganglion cells in guinea-pig	Fig. 4E,F
Calretinin	4% *p*-formaldehyde	as above	Fig. 4K,L
	sequential	as above	Fig. 2D
CGRP	4% *p*-formaldehyde	cell bodies and fibres in guinea-pig	Fig. 3G
	Zamboni's	as above	Figs. 3H and 4H,I
Synapsin I	methanol	tertiary fibres	Fig. 4B,C
	sequential	as above	Fig. 3B,C

[a] Zamboni's solution contains 0.2% picric acid.

3. Results

3.1. Vanilloid receptor immunoreactivity

When vanilloid VR1 receptor labelling with antibodies from rabbit and goat were compared, a subset of immunoreactive cells and fibres were identified in all tissues (Fig. 1A–I). Differences in immunoreactivity were detected depending on the method of fixation and vanilloid VR1 receptor antibodies. Incomplete identification was found with paraformaldehyde, methanol or modified Zamboni's fixatives. Methanol fixation was a crucial step for comprehensive labelling of vanilloid VR1 receptor-immunoreactive fibres. In methanol-fixed tissues, a few cell bodies were visible in the guinea-pig ileum (Fig. 1B) and colon (Fig. 1D,F,G) but were rarely seen in the rat ileum (Fig. 1H). Some vanilloid VR1 receptor-immunoreactive cell bodies were visible in tissues fixed in modified Zamboni's solution. In guinea-pig tissues, the most comprehensive labelling with rabbit anti-VR1 receptor antibody was achieved after sequential fixation. This procedure reduced background staining and revealed vanilloid VR1 receptor-immunoreactive perikarya and long, fine fibres running in ileal ganglia and interconnecting strands (Fig. 1A). Similar

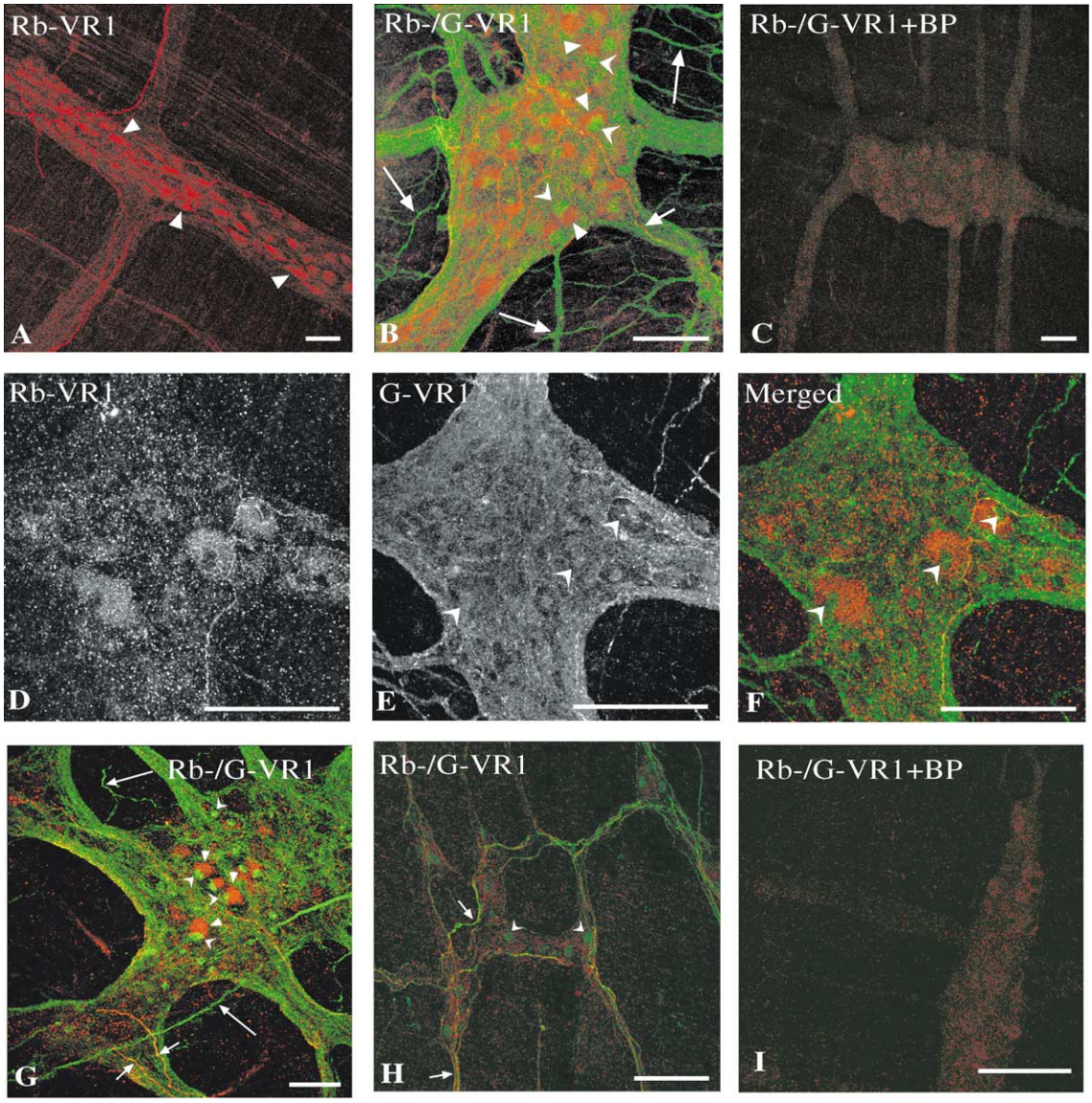

Fig. 1. Representative photomicrographs from at least three different experiments of whole-mounts of myenteric plexus-longitudinal muscle of guinea-pig ileum (A–C, I) or colon (D–G) and rat ileum (H) that were fixed sequentially (A) or with methanol (B–I), and labelled with goat (green; G-VR1) and/or rabbit (red; Rb-VR1) anti-vanilloid VR1 receptor antibodies. Cell bodies (e.g. arrowheads) were labelled with the rabbit antibody and cell 'nuclei' (e.g. concave arrowheads) with the goat antibody. Fibres that were labelled with both antibodies co-localised in the ganglia and interconnecting strands (short arrows). Another set of fibres was labelled in the tertiary plexus of the guinea-pig ileum (B) and colon (G) with the goat antibody (long arrows). Ileal tissue that was pre-absorbed with vanilloid VR1 receptor blocking peptide (BP) lacked immunoreactivity for both antibodies (Novartis blocking protein, C; Santa Cruz blocking protein, I). Scale bars are 50 μm.

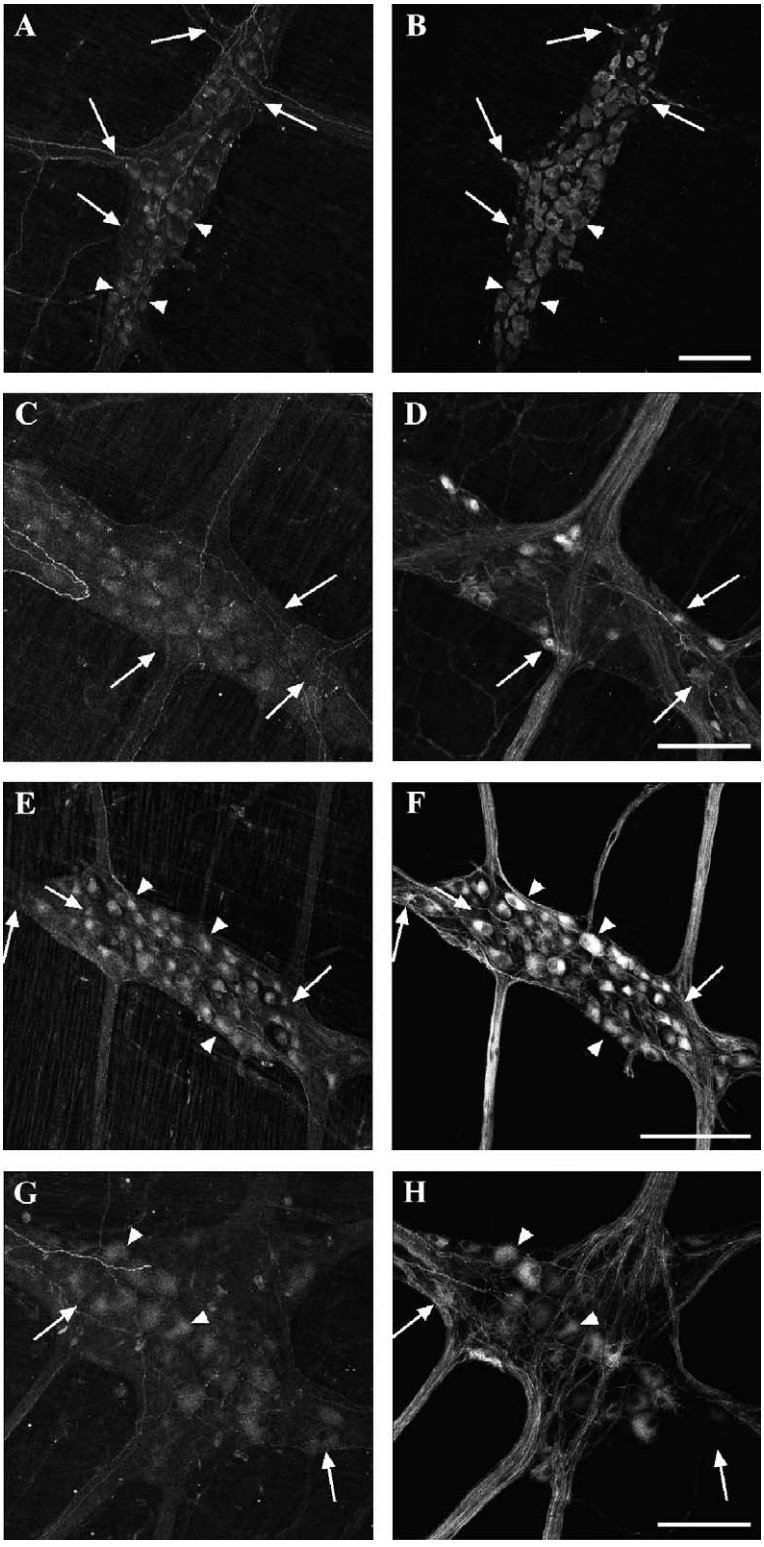

Fig. 2. Pairs of corresponding photomicrographs of myenteric ganglia demonstrating localisation of vanilloid VR1 receptor-immunoreactive cells (A, C, E, G; Rb-VR1) with choline acetyltransferase (B), calretinin (D), NFP-200 (F) or NFT (H) in the guinea-pig ileum (A–F) and colon (G, H). In A and B, all vanilloid VR1 receptor-immunoreactive cell bodies were co-localised with choline acetyltransferase (e.g. arrowheads) but some cells were cholinergic only (arrows). The majority of calretinin cells were vanilloid VR1 receptor-negative (arrows in C, D). In E–H, a proportion of vanilloid VR1 receptor-immunoreactive cells was immunoreactive for neurofilament proteins (e.g. arrowheads). However, some cells (E–H) were not co-labelled (e.g. arrows). Representative images are from three different experiments. Scale bars are 100 μm.

immunoreactivity was observed in the colon (see below) and rat ileum (not illustrated) (Anavi Goffer et al., 2002). Therefore, with the rabbit antibody, a sequential fixation was used for dual labelling unless the specific antibody for the co-label did not stain well after methanol. The goat anti-VR1 receptor antibody labelled a subset of myenteric cell nuclei (Fig. 1B,E–H) and fine fibres in the ganglia and interconnecting strands (Fig. 1B,E–H). In the guinea-pig ileum, the goat antibody labelled an additional network of fibres in the tertiary plexus (Fig. 1B) that was less developed in the colon (Fig. 1E,G). Labelling of vanilloid VR1 receptor-immunoreactive fibres was similar with all other fixatives. However, labelling of cell nuclei, similar to that demonstrated in Fig. 1B,E–H, was observed best in paraformaldehyde-fixed tissues. Applying sequential fixation did not produce any further immunoreactivity. Comparison of vanilloid VR1 receptor immunoreactivities obtained with the two antibodies in the guinea-pig methanol-fixed tissues revealed that, in the guinea-pig ileum (Fig. 1B) and colon (Fig. 1G), vanilloid VR1 receptor-immunoreactive nuclei (goat antibody) were closely associated with, but were more abundant than vanilloid VR1 receptor-immunoreactive cell perikarya (rabbit antibody) (Fig. 1B,D,F). Some fine fibres in the primary and secondary plexuses co-localised but the tertiary fibres did not (Fig. 1B,G). In contrast, in the rat ileum, vanilloid VR1 receptor-immunoreactive fibres with both antibodies co-localised in all parts of the plexus and no evidence was found for a tertiary network (Fig. 1H). These results are summarised in Table 3. The blocking protein from Novartis was found to block all labelling with both vanilloid VR1 receptor antibodies, either singly or in combination, on fibres and nuclei and greatly reduce that on cell bodies (Fig. 1C). The same effect was found after pre-absorption with the Santa Cruz blocking protein (Fig. 1I).

3.2. Dual labelling of vanilloid VR1 receptor-immunoreactive cells

Cholinergic neurones were identified with an antibody raised against choline acetyltransferase (Fig. 2B). This antibody did not label fibres but enabled us to count cholinergic somata. The following results refer to procedures in which the rabbit antibody was used. In the guinea-pig ileum, 100% of vanilloid VR1 receptor-immunoreactive cell bodies were choline acetyltransferase-positive but only half of the cholinergic neurones were vanilloid VR1 receptor-immunoreactive (Fig. 2A,B; Table 4). In the colon, all vanilloid VR1 receptor-immunoreactive cells were also cholinergic and had large, smooth cell bodies (not illustrated). The calretinin antibody labelled Dogiel type I neurones and some cells without distinguishable processes that were less immunoreactive. Calretinin-positive cells were often located close to the periphery of the myenteric ganglia or clustered in small groups, and fibres arising from these cells formed a fibre network in the tertiary plexus of the guinea-pig ileum (Fig. 2D) but not in the colon. Compared with vanilloid VR1 receptor-immunoreactive neurones (Fig. 2C), the proportion of calretinin-immunoreactive cells was smaller and very few cells were vanilloid VR1 receptor/calretinin-immunoreactive (Fig. 2C,D). In the colon, no somatic co-labelling was observed (not illustrated). In both tissues, no co-localisation was found between calretinin and vanilloid VR1 receptors in fibres. Dual labelling with antibodies raised against neurofilament proteins enabled us to visualise subpopulations of myenteric neurones (Costa et al., 1996). Since both neurofilament antibodies were raised in the mouse, it was not possible to compare their distributions directly. In guinea-pig preparations, a larger proportion of myenteric neurones was labelled with NFP-200 compared with NFT (Fig. 2F,H). In the ileum, nearly 80% of vanilloid VR1 receptor-immunoreactive neurones co-localised with NFP-200 and a similar proportion of NFP-200-positive neurones was co-labelled with vanilloid VR1 receptor antibody (Fig. 2E,F; Table 4). Large NFP-200-positive neurones resembling Dogiel type II morphology co-localised with vanilloid VR1 receptor antibody. Some small and large Dogiel type I and large, smooth neurones were vanilloid VR1 receptor-negative. In the colon, a variety of large and small vanilloid VR1 receptor-immunoreactive cells co-localised with round or spindle-shaped NFP-200-positive neurones (not illustrated). A significantly lower proportion of vanilloid VR1 receptor/NFT-immunoreactive cells was labelled in the guinea-pig ileum (Table 4). NFT labelled some bright Dogiel type I neurones but also other cells with a lower intensity and indistinct morphology. A proportion of the

Table 4
The proportion of myenteric cells co-labelled with vanilloid VR1 receptor and choline acetyltransferase, NFP-200 or NFT antibodies in the guinea-pig ileum

Marker	Number of marker-labelled cells	Number of vanilloid VR1 receptor-labelled cells	Percentage of vanilloid VR1 receptor cells co-labelled with marker (%)	Percentage of marker cells co-labelled with vanilloid VR1 receptor (%)
Choline acetyltransferase	1079	504	100.00 ± 0.0	47.1 ± 2.8
NFP-200	481	487	79.0 ± 1.3	80.4 ± 3.9
NFT	157	367	10.8 ± 1.9[a]	24.5 ± 5.7[b]

The means and S.E.M. of percentage of co-labelled cells with combination of vanilloid VR1 receptor/choline acetyltransferase, vanilloid VR1 receptor/NFP-200 or vanilloid VR1 receptor/NFT antibodies. Cells were counted from four to six fields per specimen from three animals.

[a] The percentage of vanilloid VR1 receptor-immunoreactive cells co-localised with NFT was significantly lower than with NFP-200 ($P < 0.0001$). Comparisons were made by Student's unpaired *t*-test, for three experiments, GraphPad Prism.

[b] The percentage of NFT-immunoreactive cells co-labelled with vanilloid VR1 receptor antibody was significantly lower than NFP-200/vanilloid VR1 receptor ($P = 0.0013$). Comparisons were made by Student's unpaired *t*-test, for three experiments, GraphPad Prism.

latter group co-localised with vanilloid VR1 receptor, and the brightly labelled NFT-positive neurones were predominantly vanilloid VR1 receptor-negative. In the colon, results were similar (Fig. 2G,H). In both tissues, neurofilament protein-labelled fibres did not co-distribute with vanilloid VR1 receptor-immunoreactive fibres.

3.3. Localisation of vanilloid VR1 receptor-immunoreactive fibres

To determine whether vanilloid VR1 receptors are localised at sites that may be involved in the release of neuropeptides and neurotransmitters, guinea-pig myenteric

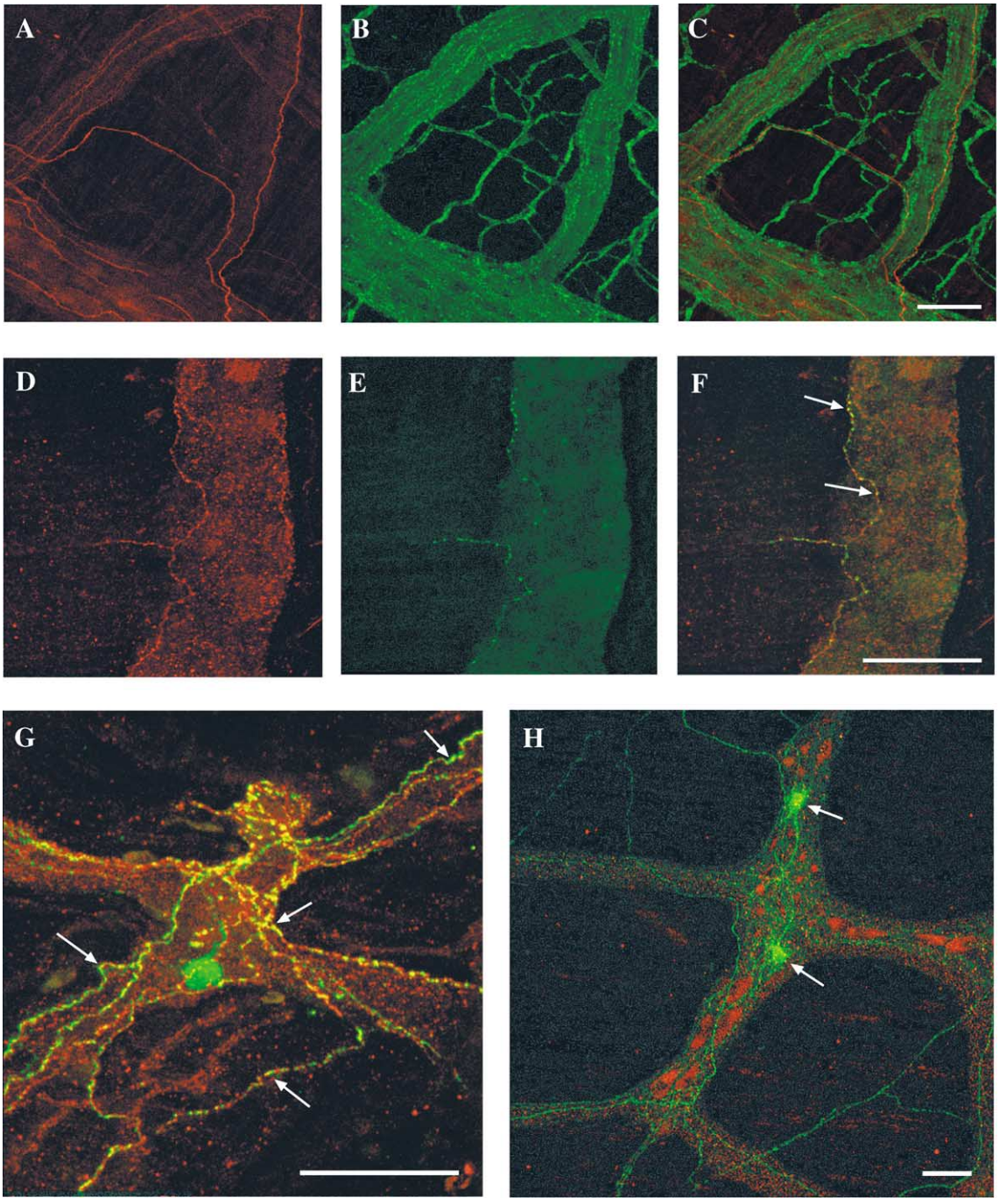

Fig. 3. Typical photomicrographs from three different experiments of guinea-pig myenteric plexus-longitudinal muscle ileal preparations showing localisation of vanilloid VR1 receptor-immunoreactive fibres labelled with the rabbit antibody (A, C, D, F, G, H; Rb-VR1) with synapsin I (B, C), substance P (E, F) and CGRP (G, H). The merged images (C, F) show co-localisation (arrows) of vanilloid VR1 receptor-immunoreactive fibres with substance P-immunoreactive fibres (F) but not with synaptic protein (C). Rabbit anti-VR1 receptor antibody labelled fibres in paraformaldehyde-fixed tissues (G) and cell bodies in modified Zamboni's-fixed tissues (H). For example, arrows in G–H show vanilloid VR1 receptor-immunoreactive fibres (G) and cells (H) that were also immunoreactive to CGRP. D–F are images of a single section. Scale bars are 50 μm.

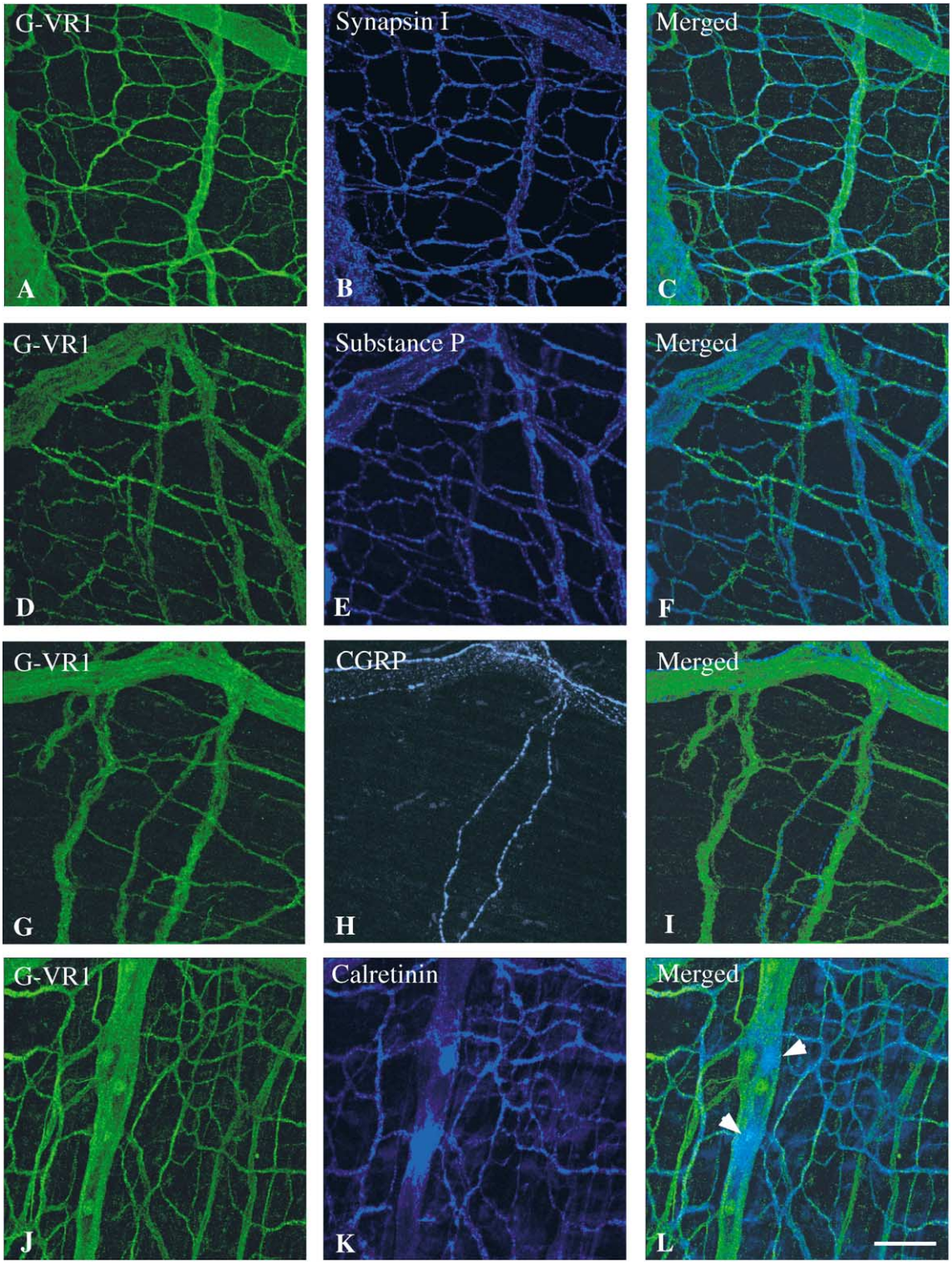

Fig. 4. Corresponding photomicrographs of vanilloid VR1 receptor-immunoreactive fibres in guinea-pig myenteric plexus-longitudinal muscle ileal preparations that were dual-labelled with goat anti-VR1 receptor antibody (A, D, G, J; G-VR1) and with synapsin I (B), substance P (E), CGRP (H) or calretinin (K). The goat antibody labelled a varicose network of vanilloid VR1 receptor-immunoreactive fibres in the tertiary plexus in tissues that were fixed with methanol (A), modified Zamboni's (D, G) or paraformaldehyde (J) fixatives. Vanilloid VR1 receptor-immunoreactive nuclei were labelled after methanol or paraformaldehyde (J) fixation. The merged images (C, F, I, L) show co-distribution of vanilloid VR1 receptor-immunoreactive varicose fibres in the tertiary plexus with synapsin I- (C), substance P- (F) and calretinin- (L) immunoreactive fibres but not with CGRP-immunoreactive fibres (I). Few vanilloid VR1 receptor-immunoreactive nuclei were co-labelled with calretinin (arrowheads in L). A–I are representative images from three different experiments. J–L are representative images from two different experiments. Scale bar is 50 μm for all images.

preparations were dual-labelled with either of the vanilloid VR1 receptor antibodies and with CGRP, substance P or synapsin I. Synapsin I identified synaptic terminals on punctate ascending and descending fibres, also surrounding ganglion cells and forming a varicose tertiary network (Fig. 3B, rabbit vanilloid VR1 antibody; Fig. 4B, goat vanilloid VR1 antibody). Vanilloid VR1 receptor-immunoreactive fibres in the primary and secondary plexus that were labelled with the rabbit antibody (Fig. 3A) did not co-localise with synapsin I but fine fibres in the tertiary plexus distributed closely, thus entwining synapsin I-positive fibres (Fig. 3C). On the other hand, vanilloid VR1 receptor-immunoreactive varicose fibres labelled with the goat antibody (Fig. 4A) co-distributed with synapsin I presumably at synaptic terminals (Fig. 4C). Fibres were labelled with substance P after methanol (Fig. 3E) or in modified Zamboni's fixative (Fig. 4E). Substance P was located in some ganglion cells (not illustrated) and fine, punctate fibres along the interganglionic strands. In addition, a network of fine fibres in the tertiary plexus was immunoreactive and resembled the anastamosing network seen with the goat anti-VR1 receptor antibody. The merged images (Fig. 3F, rabbit vanilloid VR1 antibody; Fig. 4F, goat vanilloid VR1 antibody) demonstrate a close association of vanilloid VR1 receptor/substance P-immunoreactive fibres and co-localisation of some individual puncta in the tertiary plexus. A similar co-localisation of fibres was found in colonic preparations (not illustrated). In ileal tissues dual-labelled with vanilloid VR1 receptor and CGRP antibodies, cell bodies and individual punctate fibres pervading all plexuses were detected with the CGRP antibody (Figs. 3G,H and 4H). Some cells and fibres that were labelled with the rabbit vanilloid VR1 receptor antibody co-localised with CGRP-immunoreactive neurones and fibres (Fig. 3G,H), whereas no association was found between CGRP and goat vanilloid VR1 receptor-immunoreactive fibres (Fig. 4I) although a proportion of vanilloid VR1 receptor-immunoreactive nuclei (goat vanilloid VR1 antibody) were co-labelled with CGRP-positive cells (not illustrated). The distribution of calretinin-positive fibres was compared with that of goat vanilloid VR1 receptor-immunoreactive fibres in the tertiary plexus. The majority of vanilloid VR1 receptor-immunoreactive nuclei were not associated with calretinin-positive cells (Fig. 4L). In contrast, varicose calretinin-immunoreactive fibres co-distributed with vanilloid VR1 receptor-immunoreactive fibres in the tertiary plexus (Fig. 4L).

4. Discussion

A population of myenteric cells and fibres bearing vanilloid VR1 receptors were identified in myenteric plexus-longitudinal muscle whole-mounts of guinea-pig ileum and proximal colon and rat ileum, thus confirming our earlier report (Anavi-Goffer et al., 2002). The presence of vanilloid VR1 receptors on both perikarya and fibres concurs with findings in the pig (Poonyachoti et al., 2002). Specific labelling of vanilloid VR1 receptors in all tissues suggests that the amino acid sequence of the vanilloid VR1 receptor peptide is similar in the intestine of both species, as has been confirmed recently by cloning (Savidge et al., 2002). However, vanilloid VR1 receptors were identified in different neuronal compartments depending on the fixation method and antibody that were used. These findings are of concern to us and raise the possibility that some of the staining may not be specifically related to vanilloid VR1 receptor protein. Alternatively, despite the fact that both polyclonal antibodies were raised against the same amino acid sequence, it may be that, when administered to the different hosts, the immunising peptide existed in slightly different tertiary conformations, thus giving rise to different polyclonal antibody combinations and different staining patterns. Also, the guinea-pig vanilloid VR1 receptor phenotype differs slightly from that of the rat (Anavi-Goffer et al., 2002; Savidge et al., 2002). However, the fact that labelling with both vanilloid VR1 receptor antibodies was blocked by preincubation with the blocking protein from either antibody sources and that both antisera labelled some of the same structures led us to believe that the labelling was specific. Under certain fixation conditions, the antigen(s) may be masked and therefore not available for binding. An interesting finding was the variation in distribution of vanilloid VR1 receptors between species. Our conclusions are based on the results obtained with the procedures described, but we accept that there may be possible limitations to our methods. Previous studies of guinea-pig and human gut suggest an extrinsic origin of capsaicin-sensitive and vanilloid VR1 receptor-expressing fibres (Barthó and Szolcsányi, 1978; Yiangou et al., 2001), while a recent study demonstrated that some porcine myenteric cells express vanilloid VR1 receptors (Kulkarni-Narla and Brown, 2001; Poonyachoti et al., 2002). In view of these studies, it may be that the differences in vanilloid VR1 receptor immunoreactivity in the guinea-pig with different antibodies in our study may be related to the origin of the fibres. As fibres of both origins may be present in our preparations, it would be useful to label chronically deafferented intestinal tissues with the same antibodies to exclude their extrinsic nervous supply. The difference in labelling in the guinea-pig may be due to the detection of a closely related protein or a splice variant of vanilloid VR1 receptor (Sanchez et al., 2001). Clearly, this needs further investigation.

Guinea-pig myenteric neurones may be classified into subpopulations according to their neurochemical coding, morphology and electrophysiological properties (Costa et al., 1996; Lomax and Furness, 2000; Coutts et al., 2002). Since we found that all vanilloid VR1 receptor-expressing cells were cholinergic, then according to this classification these cells would be excitatory. This supports similar findings in the porcine preparations (Poonyachoti et al.,

2002). Although it is not clear if the actions of capsaicin in the gut are vanilloid VR1 receptor-mediated, the current findings are in line with earlier studies indicating that acetylcholine is the main final neurotransmitter in some of the responses to capsaicin (Barthó et al., 1999; Barthó and Szolcsányi, 1978; Mang et al., 2001). Previous studies suggest that the capsaicin-evoked contractile response of the longitudinal muscle (Barthó et al., 1982) and of the circular muscle (Bartho et al., 1994) involves the release of substance P from motor neurones themselves or from capsaicin-sensitive primary afferent neurones (Barthó et al., 1999).

The excitatory motoneurones are characterised with substance P and/or calretinin immunoreactivity (Costa et al., 1996; Lomax and Furness, 2000). Co-distribution of vanilloid VR1 receptor-immunoreactive fibres in the tertiary plexus with substance P and calretinin implies that vanilloid VR1 receptor could be expressed on excitatory motoneuronal fibres. Further support for this hypothesis comes from functional and immunohistochemical studies demonstrating that these neurones express both vanilloid and cannabinoid CB_1 receptors that modulate acetylcholine release (Coutts et al., 2002; Mang et al., 2001).

NFT is thought to be expressed by subpopulations of interneurones (Costa et al., 1996; Lomax and Furness, 2000). As a small percentage of myenteric cells co-expresses vanilloid VR1 receptor/NFT, it is reasonable to assume that only a small proportion of interneurones expresses vanilloid VR1 receptors. This supports a previous study demonstrating a partial resistance of the contractile response to capsaicin following blockage of ganglionic transmission (Barthó and Szolcsányi, 1978). On the other hand, a larger population of cells co-expresses vanilloid VR1 receptor and NFP. Although both NFT and NFP can label neurofilament protein of a similar molecular weight (Vitadello et al., 1986), it is clear that the commercially available antibody, NFP, labels a bigger proportion of myenteric neurones than previously classified with NFT (Costa et al., 1996). As the majority of vanilloid VR1 receptor-expressing cells were also immunoreactive to NFP, it would be useful to ascribe NFP immunoreactivity to some of the other neurochemical populations as described by Costa et al. (1996). We have recently demonstrated that nearly 68% of vanilloid VR1 receptor-positive neurones in the guinea-pig ileum, the majority of which appear to be Dogiel type II, also express calbindin, a marker for sensory neurones. Since approximately 80% of vanilloid VR1 receptor-expressing cells also express NFP, then the chemical identity of at least 50% of these sensory neurones would be choline acetyltransferase/vanilloid VR1 receptor/NFP/calbindin/ ± substance P. These findings agree with recent reports of vanilloid VR1 receptor expression on primary sensory neurones in porcine enteric plexuses (Poonyachoti et al., 2002) and support previous studies of a cholinergic component involved in axon reflex in the actions of capsaicin at terminals of primary afferent neurones (Barthó et al., 1999). However, calbindin-positive neurones account for only 24.6% of all ileal myenteric neurones (Costa et al., 1996); therefore, it is likely that the vanilloid VR1 receptor antibody labels more than one subpopulation of neurones. In our preparations, some cells co-expressed vanilloid VR1 receptor and CGRP. According to Costa et al. (1996), myenteric neurones innervating the mucosal secretory cells are the only class of neurones that express both choline acetyltransferase and CGRP and therefore may express vanilloid VR1 receptor, although these are thought to account for only 1% of the myenteric population. However, in the guinea-pig colon, choline acetyltransferase/CGRP are co-expressed by descending interneurones (Lomax and Furness, 2000). Moreover, in our study, only vanilloid VR1 receptor-immunoreactive fibres labelled with the rabbit antibody co-localised with CGRP. This supports the modulation of CGRP released by capsaicin from capsaicin-sensitive fibres (Barthó et al., 1991; Rasmussen et al., 2001) but again raises the significance of the source of vanilloid VR1 receptor fibres.

It is tempting to suggest that vanilloid VR1 receptors which are found in the tertiary plexus may modulate the synaptic release of both neurotransmitters and neuropeptides from excitatory motor neurones.

In conclusion, this investigation and comparison of the immunoreactivity of vanilloid VR1 receptor antibodies has contributed to our knowledge of the cellular distribution of vanilloid VR1 receptor in the myenteric plexus. In the guinea-pig tissues, the results obtained with our procedures suggest that vanilloid VR1 receptor-expressing neurones were cholinergic excitatory neurones. These are likely to be sensory, interneuronal and ‘secretory’ neurones. Co-expression of vanilloid VR1 receptor together with substance P, calretinin and synapsin I in the tertiary plexus and with CGRP in the ganglia and interganglionic strands corroborate previous functional studies of the actions of capsaicin on the intestine. They support the hypothesis that a receptor-mediated mechanism could be involved in the capsaicin-induced release of neuropeptides and neurotransmitters. In the guinea-pig, at least two forms of vanilloid VR1 receptor-immunoreactive fibres were labelled, whereas in the rat ileum, both antibodies identified the same system. The physiological significance of vanilloid VR1 receptors in the intestine and their role in gastrointestinal pathology are still to be elucidated.

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