

Short communication

Interstitial cells of Cajal and purinergic signalling

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Abstract

Interstitial cells of Cajal (ICC) in the guinea pig intestine, identified by the tyrosine kinase receptor, c-Kit, have been shown with immunohistochemistry to express nucleotide P2X₂ and P2X₅ receptors. P2X₅ receptors have also been demonstrated on interstitial cells in the mouse ileum. It is speculated that release of ATP from enteric nerves, enteric glial cells or from contracting smooth muscle may provide a feedback mechanism for pacemaker activity in the intestine. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Interstitial cells of Cajal (ICC) are a special class of cells dispersed in the muscle and nerve plexuses of the gastrointestinal tract (Cajal, 1893; Komuro et al., 1996). These cells are of mesenchymal origin and play major roles in gastrointestinal motility (see Ward and Sanders, 2001). They are now well established as the pacemaker cells for the spontaneous contractions of the smooth muscle coats (Thunberg, 1982; Huizinga, 1999). ICC are also responsible for the active propagation of electrical slow waves (Dickens et al., 2001) and have been claimed to mediate motor inputs from enteric nerves (Ward and Sanders, 2001). NK₁ receptors for substance P (Portbury et al., 1996), muscarinic M₂ and M₃ receptors for acetylcholine (Epperson et al., 2000), and somatostatin 2A receptors (Sternini et al., 1997) have been identified on ICCs.

In the present study, we describe the expression of P2X₂ and/or P2X₅ receptors on ICCs, identified with the tyrosine kinase receptor, c-Kit (Maeda et al., 1992), in the guinea pig and mouse ileum and discuss the possibility that release of ATP from enteric nerves, enteric glial cells or during spon-

aneous contractions of smooth muscle provides a feedback mechanism for pacemaker activity in the gut.

2. Materials and methods

2.1. Animals

Breeding, maintenance, and killing of the animals used in this study followed the principles of good laboratory animal care and experimentation in compliance with the UK national law and regulations. Experiments were carried out on male guinea pigs at 2 weeks and 1 year and on mature male mice. Animals were killed by a rising concentration of CO₂ and death was confirmed by cervical dislocation. Subsequently, animals were perfused, through the heart, with 4% formaldehyde (in 0.1 M phosphate buffer) containing 0.2% of a saturated solution of picric acid (pH 7.4).

2.2. Guinea pig wholemount preparations

Guinea pig proximal ileum was collected in ice-cold phosphate-buffered saline (PBS). An incision was made along the midline of the gut and the tissue was pinned back into a flat sheet on Sylgard (Dow Corning, Weisbaden, Germany) with the mucosa faced down. Tissue samples were fixed overnight in 4% formaldehyde (10% formalin in 0.1 M phosphate buffer), unpinned, and washed in PBS (3 × 10 min). The mucosa, sub-mucosal plexus (SMP), and circular muscle

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layers were removed to leave the myenteric plexus (MP) and longitudinal muscle layers. Wholemount, 1-cm² preparations, were prepared from MP for immunohistochemistry.

2.3. Guinea-pig and mouse sections

Guinea pig and mouse ileum was collected in ice-cold PBS and lumens flushed with PBS. Tissue was mounted using Tissue Tek (Sakura, Zoeterwoude, Netherlands), corked and progressively frozen in isopentane (pre-cooled in liquid nitrogen), and then stored in liquid nitrogen. Cryostat sections were cut at 20- μ m thickness. The sections were thaw-mounted on gelatine-coated slides and air-dried

at room temperature for 1 h. Tissues were post-fixed for 2 min at room temperature in 4% formaldehyde (BDH Laboratory Supply, Poole, UK) and 0.03% picric acid (BDH) in PBS, and then washed in PBS for 3×10 min.

2.4. Immunohistochemistry

For immunolabelling of P2X receptors, in both guinea pig and mouse ileum, an indirect immunofluorescence method was used. Antibodies for P2X₁–P2X₇ receptors from rabbit were allowed to react with biotinylated donkey anti-rabbit IgG secondary antibody (Jackson Immuno-research, Pennsylvania, USA) and detected with streptavidin-

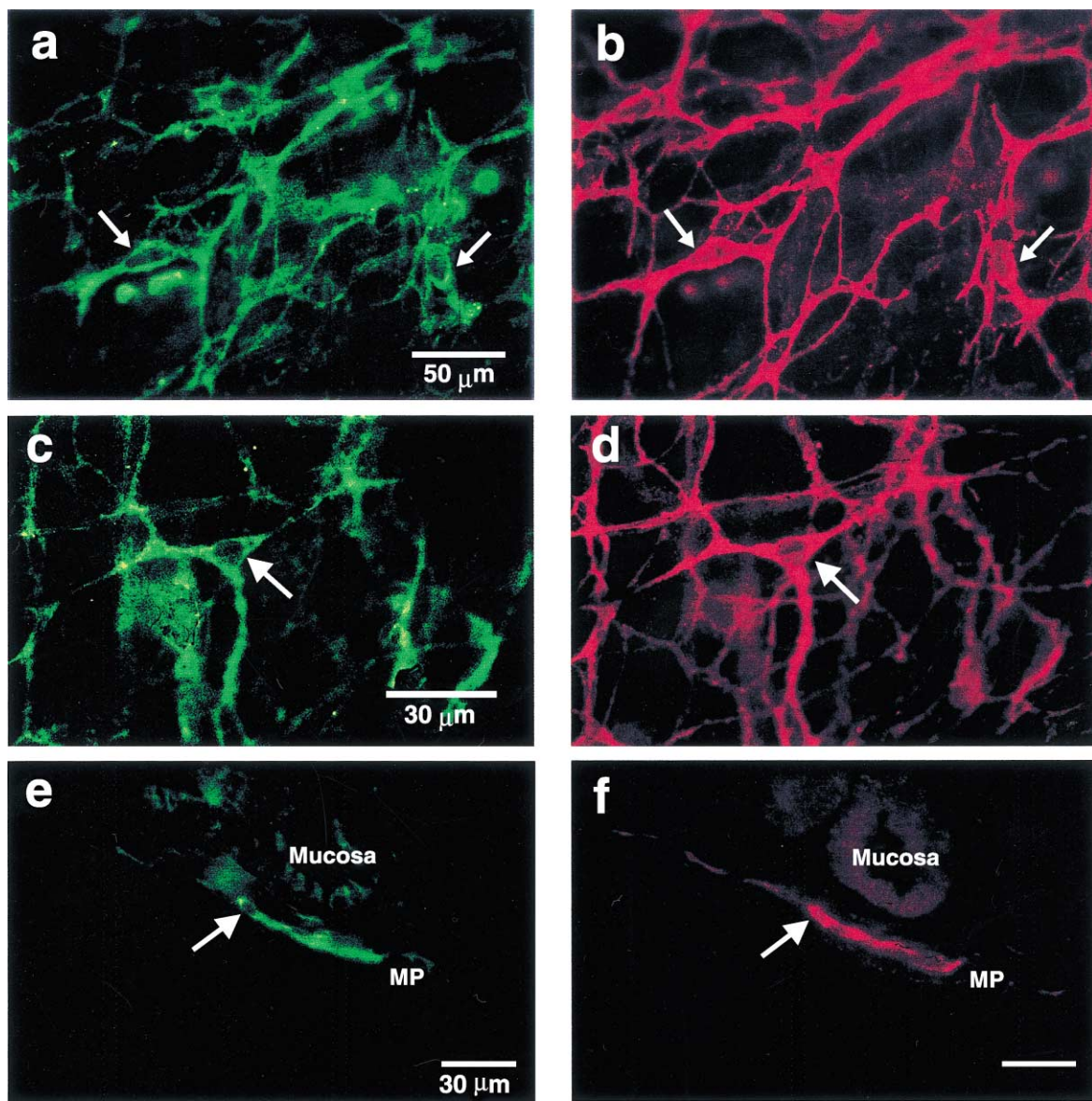


Fig. 1. P2X₂ (a) and P2X₅ (c and e) receptor immunolabelling of interstitial cells of Cajal (arrows), double-labelled with c-Kit (arrows) in wholemount myenteric plexus preparations (b and d) of guinea pig ileum (a–d) and sections of submucosal plexus of mouse ileum, with c-Kit labelling (e) and P2X₅ receptor labelling (f) indicated by arrows. MP, myenteric plexus.

FITC (Amersham Pharmacia Biotech UK, Little Chalfont, UK). The P2X antibodies were obtained from Roche Bioscience (Palo Alto, CA, USA). P2X sub-type selective antibodies were each raised in rabbits against a specific 15 amino acid residue at the carboxy-terminus of each P2X receptor molecule (Oglesby et al., 1999).

For immunolabelling of c-Kit, a goat anti-c-Kit antibody (Santa Cruz International, California, USA) was allowed to react with a directly labelled anti-goat conjugated Cy3 antibody (Jackson ImmunoResearch, PA, USA). Since the primary antibodies were raised in different species and were shown not to cross-react, the immunolabelling for P2X and c-Kit procedures were carried out simultaneously.

Briefly, blocking of non-specific binding sites was achieved by pre-incubation of wholemounts or sections of tissue with 10% normal horse serum (NHS; Harlan Sera-Lab, Loughborough, UK), 0.1% Triton (Sigma, Dorset, UK) in PBS for 60 min. Wholemounts/sections were incubated overnight in anti-P2X antibodies (1:200) and goat anti-c-Kit (1:500; antibodies were diluted in the blocking serum above). Subsequently, slides were incubated with biotinylated donkey anti-rabbit IgG diluted 1:500 in 1% NHS, 0.1% Triton in PBS for 90 mins. All incubations were carried out at room temperature and separated by three 5-min washes in PBS. Finally, slides were incubated in Strep-FITC (1:200) diluted in 1% NHS, 0.1% Triton in PBS and donkey anti-goat conjugated Cy3 (1:500) diluted in 1% NHS, 0.1% Triton in PBS for 60 min. Tissue was washed 3×10 min in PBS, mounted in CitiFluor (Citifluor, London, UK) and viewed.

2.5. Controls

The following control experiments were performed to establish the specificity of immunoreaction: omission of the primary antibodies; replacement of the primary antibodies with rabbit pre-immune IgG or a non-reactive P2X; and duplicate absorptions of the primary antibodies with their homologous peptide antigen.

2.6. Microscopy

The wholemounts or sections were viewed using an Edge True-view 3D fluorescence microscope (Edge Scientific Instruments, Santa Monica, CA, USA) with Kodak Ektachrome400 colour film. P2X immunoreactivity was observed using the blue filter (FITC = green) and c-Kit immunoreactivity using the green filter (Cy3 = red).

3. Results

ICCs are classified into four groups based on their morphology and distribution within the different layers of the gastrointestinal tract (Komuro et al., 1996). In this study, we focussed on the type of ICC population that is located at

the level of the myenteric plexus (ICC-MP). The ICC-MP branches out to form a network within the plane of the MP that is closely associated with neurons and adjacent smooth muscle cells.

When wholemounts of guinea pig tissue were incubated with the P2X antibodies, immunopositive ICC-like cells were observed at the level of the MP. These cells had small oval-shaped bodies with little cytoplasm and long branching processes that are connected together to form a large network that is characteristic of ICCs.

The cell-specific marker for ICCs within the gastrointestinal tract, tyrosine kinase receptor known as c-Kit, was used to determine whether the ICC-like cells that were observed were, in fact, true ICC. Double-labelled tissue with c-Kit and P2X antibodies showed that there was P2X₂- and P2X₅-IR on ICCs from the MP of the guinea pig. Wholemounts from 2 week and 1-year-old guinea pigs both showed P2X₂ and P2X₅-labelled ICCs. No immunostaining for P2X₁, P2X₃, P2X₄, P2X₆ or P2X₇ was observed in ICCs.

The c-Kit-IR in the double-labelled preparations revealed a fine network of interconnecting ICC at the level of the myenteric plexus (Fig. 1b and d). Corresponding P2X-IR was present for P2X₂ (Fig. 1a) and P2X₅ (Fig. 1c). Control experiments described in the Materials and methods were negative for P2X receptor immunostaining.

Double labelling of 20- μ m sections of mouse ileum with c-Kit and P2X₅ also showed positive ICCs at the level of the myenteric plexus (Fig. 1e and f).

4. Discussion

Unambiguous identification of ICC is now possible. In early papers, supravital methylene blue staining was used (Cajal, 1893; Thuneberg, 1982). However, this staining was usually incomplete such that uptake of dye was restricted to patches of the ICC network, although a modification was introduced that claimed 'nearly selective' staining of the entire ICC network by brief exposure of the intestine to lyssolecithin prior to vital methylene blue staining (Mikkelsen et al., 1988). The Champy–Maillet stain has also been claimed to reveal ICC (Christensen et al., 1987), as well as S-100 protein (Kobayashi et al., 1989). Currently, the most widely accepted marker for ICCs is the receptor tyrosine kinase, c-Kit, which has been used to examine the distribution of ICCs in the guinea pig intestine (Burns et al., 1997; Wang et al., 1999), which show similar distribution in our studies.

A question that is raised by the presence of P2X₂ and P2X₅ receptors in ICCs is what is the source of ATP that would act on these receptors? One possibility is that ATP is a cotransmitter with ACh, noradrenaline or substance P in nerve terminals that are known to form close association with ICCs (Huizinga, 1999; Wang et al., 1999). ATP has been shown to be released from nerve varicosities isolated from the myenteric plexus of the guinea pig ileum (Al

Humayyd and White, 1985) and ATP is known to be a cotransmitter in most nerves in both the peripheral and central nervous systems (see Burnstock, 1976, 1990; Abbraccio, 1997). Other possibilities are that ATP is released from smooth muscle cells during spontaneous contractions (Katsuragi et al., 1996) or from enteric glial cells, since these cells, like astrocytic glia in the CNS, stain with glial fibrillary acidic protein (GFAP) (Jessen and Burnstock, 1982) and astrocytes are known to release ATP (Guthrie et al., 1999). This raises the interesting possibility that ATP released from enteric nerves, enteric glial cells or during smooth muscle contractions provides a feedback system for ICCs to modulate slow wave activity.

What is known about the functions of P2X₂ and P2X₅ receptors? P2X₂ receptors are widely distributed in the nervous system and appear to mediate fast neurotransmission (Dunn et al., 2001; Khakh et al., 2001). The only clear indication of function for P2X₅ receptors comes from studies showing labelling during differentiation of skin keratinocytes (Gröschel-Stewart et al., 1999a) and mucosal epithelial cells during turnover in gut and bladder (Gröschel-Stewart et al., 1999b; Lee et al., 2000). They have also been localised on neurones in trigeminal, nodose, and dorsal root ganglia (Xiang et al., 1998), and in developing neurones and myotubes in embryos (Bo et al., 2000; Ryten et al., 2001). Occupation of both P2X₂ and P2X₅ receptors leads to an increase in intracellular [Ca²⁺]_i and this would probably result in the modulation of slow wave activity. There are no selective agonists or antagonists for P2X₂ and P2X₅ receptors, but 2-methylthio ATP and ATPγS, respectively, have potent agonist actions and suramin and PPADS have antagonist actions on both receptors (Khakh et al., 2001). It is possible that P2X₂ and P2X₅ receptors form heteromultimers (Torres et al., 1999) which would have a unique pharmacology.

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