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Localization and function of adenosine receptor subtypes at the longitudinal muscle – Myenteric plexus of the rat ileum

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ABSTRACT

Adenosine plays a dual role on acetylcholine (ACh) release from myenteric motoneurons via the activation of high-affinity inhibitory A_1 and facilitatory A_{2A} receptors. The therapeutic potential of adenosine-related compounds for controlling intestinal motility and inflammation, prompted us to investigate further the role of low-affinity adenosine receptors, A_{2B} and A_3, on electrically-evoked (5 Hz, 200 pulses) [^{3}H]ACh release from myenteric neurons. Immunolocalization studies showed that A_{2B} receptors exhibit a pattern of distribution similar to the glial cell marker, GFAP. Regarding A_1 and A_3 receptors, they are mainly distributed to cell bodies of ganglionic myenteric neurons, whereas A_{2A} receptors are localized predominantly on cholinergic nerve terminals. Using selective antagonists (DPCPX, ZM241385 and MRS1191), data indicate that modulation of evoked [^{3}H]ACh release is balanced through tonic activation of inhibitory (A_1) and facilitatory (A_{2A} and A_3) receptors by endogenous adenosine. The selective A_{2B} receptor antagonist, PSB603, alone was devoid of effect and failed to modify the inhibitory effect of NECA. The A_3 receptor agonist, 2-Cl-IB MECA (1–10 nM), concentration-dependently increased the release of [^{3}H]ACh. The effect of 2-Cl-IB MECA was attenuated by MRS1191 and by ZM241385, which selectively block respectively A_3 and A_{2A} receptors. In contrast to 2-Cl-IB MECA, activation of A_{2A} receptors with CGS21680C attenuated nicotinic facilitation of ACh release induced by focal depolarization of myenteric nerve terminals in the presence of tetrodotoxin. Tandem localization of excitatory A_3 and A_{2A} receptors along myenteric neurons explains why stimulation of A_3 receptors (with 2-Cl-IB MECA) on nerve cell bodies acts cooperatively with prejunctional facilitatory A_{2A} receptors to up-regulate acetylcholine release. The results presented herein consolidate and expand the current understanding of adenosine receptor distribution and function in the myenteric plexus of the rat ileum, and should be taken into consideration for data interpretation regarding the pathophysiological implications of adenosine on intestinal motility disorders.

1. Introduction

The enteric nervous system (ENS) is organized in a complex structure that controls motility, secretion, blood flow, nutrients absorption, and immunological responses in the gut. Neurons and glial cells are the main cell populations represented in the ENS; glial cells, which stain for glial fibrillary acidic protein (GFAP), are located juxtaposition to neurons outnumbering them by a 4:1 ratio (Rühl et al., 2004; Bassoti et al., 2007). Like enteric neurons, glia express receptors for neurotransmitters and neuromodulators and so may serve as intermediates in enteric neurotransmission (Rühl et al., 2004; Gulbransen and Sharkey, 2009).

Acetylcholine (ACh) is regarded as the major excitatory neurotransmitter and prime regulator of gastrointestinal motility. The release of ACh from enteric cholinergic nerves is under well-regulated presynaptic control, involving specific membrane receptors. Among these are P1 and P2 purinoceptors, which upon

Abbreviations: ACh, acetylcholine; ADA, adenosine deaminase; CGS 21680C, 2-*p*-(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamidoadenosine hydrochloride hydrate; 2-Cl-IBMECA, 1-[2-chloro-6-[[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-1-deoxy-*N*-methyl-b-D-ribofuranuronamide; DMPP, 1,1-dimethyl-4-phenylpiperazinium iodide; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; DPM, disintegrations per min.; ENS, enteric nervous system; GFAP, glial fibrillary acidic protein; GI, gastrointestinal; IM-ICC, intramuscular interstitial cells of Cajal; LM-MP, longitudinal muscle-myenteric plexus; MRS 1191, 3-ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate; NECA, 5′-(*N*-ethylcarboxamido) adenosine; PBS, phosphate saline buffer; PGP 9.5, protein gene-product 9.5; PSB 603, 8-[4-[4-(4-chlorophenzyl)piperazide-1-sulfonyl)phenyl]]-1-propylxanthine; nAChR, nicotinic acetylcholine receptors; TTX, tetrodotoxin transporter; VAChT, vesicular acetylcholine; ZM 241385, 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol.

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activation (by adenosine and ATP, respectively) enhance or inhibit ACh release (Vizi and Knoll, 1976; Somogyi and Vizi, 1988; De Man et al., 2003; Duarte-Araújo et al., 2004a; Duarte-Araújo et al., 2009; reviewed by Ren and Bertrand, 2008).

Adenosine is a ubiquitous neuromodulator exerting its action through the activation of four distinct subtypes of P1 purinoceptors, named A_1, A_{2A}, A_{2B} and A_3. These receptors are all members of the G protein-coupled receptor family (GPCRs): A_1 and A_3 receptors generally couple to $G_{i/o}$ (although in some tissues A_3 may also couple to $G_{q/11}$), whereas A_{2A} and A_{2B} receptors couple predominantly to G_s proteins (Fredholm et al., 2001; Yaar et al., 2005; Kolachala et al., 2008). The way adenosine builds its influence to control transmitter release depends on the endogenous concentration of the nucleoside near receptor sites, which may be balanced by adenosine inactivation through cellular uptake and extracellular deamination (Duarte-Araújo et al., 2004a; Correia-de-Sá et al., 2006). Endogenous adenosine concentrations required for half-occupancy of rat A_1 and A_{2A} receptors is in the range of 10^{-8}–10^{-7} M, while the K_i value for adenosine binding to rat A_3 receptor has been estimated to be in the micromolar range (Jacobson, 1998). In view of this, A_1 and A_{2A} receptors are considered high affinity receptors (activated under basal adenosine concentrations), whereas A_{2B} and A_3 correspond to low affinity receptors in the rat which are more likely to be activated during pathological conditions as endogenous adenosine levels become elevated (Antonioli et al., 2008; Bozarov et al., 2009). Regarding A_{2B} receptors, they are always described as being a low-affinity variant of A_{2A} receptors, and are frequently found in the same tissue. The lack of selective pharmacological tools has been the main obstacle in defining the pathophysiological role of A_{2B} receptors (Feoktistov and Biaggioni, 1997).

There is an increasing interest at developing drugs that act through direct stimulation of adenosine receptors or increase local adenosine levels as promising therapeutic options for the management of intestinal motility disorders, hypoxia and inflammation, as dictated from experimental disease models in rodents. At the present, it is known that the adenosine system, including the nucleoside itself, receptors, transporters and metabolic enzymes, serves important neuromodulatory and immunoregulatory functions at the human gastrointestinal tract (reviewed by Antonioli et al., 2008). Using subtype selective adenosine receptor agonists and antagonists, our group demonstrated that adenosine exerts a dual role on ACh release from stimulated myenteric motoneurons of the rat ileum via the activation of high affinity inhibitory A_1 and facilitatory A_{2A} receptors (Duarte-Araújo et al., 2004a). We found that endogenously generated adenosine plays a predominantly tonic facilitatory effect mediated by A_{2A} receptors. Endogenous adenosine accumulation, either by increasing the number of stimulation pulses or by adding (1) the adenosine precursor AMP, (2) the adenosine kinase inhibitor 5′-iodotubercidin, or (3) inhibitors of adenosine uptake (e.g. dipyridamole) and of deamination (e.g. erythro-9(2-hydroxy-3-nonyl)adenine), enhanced [^{3}H]ACh release from myenteric nerve terminals. Data also suggest that extracellular deamination represents the most efficient mechanism regulating the levels of adenosine at the myenteric synapse. High adenosine deaminase (ADA, EC 3.5.44) activity is, therefore, the prime responsible for creating adenosine gradients from the release/production regions and for limiting diffusion of the exogenously added nucleoside towards active receptor zones at the rat myenteric plexus (Correia-de-Sá et al., 2006). Thus, the kinetics of extracellular adenosine inactivation, together with the potential nucleoside sources and regional distribution of adenosine receptors, may provide unique spatiotemporal conditions for adenosine to control excitability of the enteric nervous system. Because both adenosine and adenosine deaminase may be released from activated inflammatory cells (Marquardt et al., 1984), as well as from neighbouring neurogenic, myogenic and vascular sources (Stead et al., 1989; Bogers et al., 2000; Correia-de-Sá et al., 2006), investigation of the neuromodulatory effects exerted by adenosine during inflammatory insults and intestinal motility disorders is highly attractive. The therapeutic potential of adenosine-related compounds (designed to either activating adenosine receptors or changing the local adenosine levels) for controlling intestinal motility disorders (e.g. Akkari et al., 2006), prompted us to extend our investigations to low affinity adenosine (A_{2B} and A_3) receptors, which are more likely to be activated under pathological conditions, such as inflammation and hypoxia (see above).

Adenosine receptor subtypes are widely expressed in the human gastrointestinal tract extending from the mucosa/submucous layers to the neuromuscular compartment of both small and large intestine, as previously shown by reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemical studies (Dixon et al., 1996; Christofi et al., 2001). With regard to rodents, most of data on the distribution of adenosine receptors in rat gastrointestinal tract are based on studies designed to identify mRNA without further characterization of cellular localization (reviewed by Antonioli et al., 2008). This lack of information, prompted us to investigate the regional distribution of all four adenosine receptor subtypes in whole-mount preparations of the longitudinal muscle-myenteric plexus (LM-MP) of the rat ileum labelled with specific fluorescent antibodies by confocal microscopy.

2. Materials and methods

2.1. Preparation and experimental conditions

Rats (Wistar, 150–250 g) of either sex (Charles River, Barcelona, Spain) were kept at a constant temperature (21 °C) and a regular light (06.30–19.30 h)-dark (19.30–06.30 h) cycle, with food and water ad libitum. The animals were killed after stunning followed by exsanguination. Animal handling and experiments followed the guidelines defined by the European Communities Council Directive (86/609/EEC). A section of the rat ileum not including the terminal 5 cm was removed and the longitudinal muscle strip with the myenteric plexus attached separated from the underlying circular muscle was prepared according to Paton and Vizi (1969). This preparation is highly enriched in cholinergic neurons, mainly excitatory neurons projecting to the longitudinal muscle (25%) that receive inputs from intrinsic primary afferents (26%) and from ascending and descending pathways (17%) (Costa et al., 1996). Although ACh may also originate from preganglionic nerve endings, this represents a minor proportion of any ACh output as there is a great excess of ganglion cells in the myenteric plexus over extrinsic preganglionic fibres Paton and Vizi (1969). The experiments were performed at 37 °C in -longitudinal muscle-myenteric plexus (LM-MP) preparations mounted in a 12-chamber Brandel SF-12 system (Valley International Corp., Austin, USA) and superfused with gassed (95% O_2 and 5% CO_2) Tyrode's solution containing (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1, NaH_2PO4 0.4, $NaHCO_3$ 11.9, glucose 11.2 and choline 0.001.

2.2. [^{3}H]Acetylcholine release experiments

The procedures used for labelling the preparations and measuring evoked [^{3}H] ACh release were previously described (Duarte-Araújo et al., 2004a,b) and used with minor modifications. Longitudinal muscle-myenteric plexus (LM-MP) strips were mounted in 0.365 ml capacity perfusion chambers heated at 37 °C. After a 30-min equilibration period, nerve terminals were labelled during 40 min with 1 μM [^{3}H]choline (specific activity 5 μCi/nmol) under electrical stimulation at 1-Hz frequency