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FUNCTIONAL EXPRESSION OF P2 PURINOCEPTORS IN A PRIMARY NEUROGLIAL CELL CULTURE OF THE RAT ARCUATE NUCLEUS

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Abstract—The arcuate nucleus (ARC) plays an important role in the hypothalamic control of energy homeostasis. Expression of various purinoceptor subtypes in the rat ARC and physiological studies suggest a modulatory function of P2 receptors within the neuroglial ARC circuitry. A differentiated mixed neuronal and glial microculture was therefore established from postnatal rat ARC, revealing neuronal expression of ARC-specific transmitters involved in food intake regulation (neuropeptide Y (NPY), proopiomelanocortin (POMC), tyrosine hydroxylase (TH)). Some NPYergic neurons cosynthesized TH, while POMC and TH expression proved to be mutually exclusive. Stimulation with the general purinoceptor agonists 2-methylthioadenosine-5′triphosphate (2-MeSATP) and ATP but not the P2X1/P2X3 receptor subtype agonist α,β-methyleneadenosine-5′triphosphate (α,β-meATP) induced intracellular calcium signals in ARC neurons and astrocytes. Some 5–10% each of 2-MeSATP responsive neurons expressed POMC, NYP or TH. Supporting the calcium imaging data, radioligand binding studies to hypothalamic membranes showed high affinity for 2-MeSATP, ATP but not α,β-meATP to displace [α-^{35}S]deoxyadenosine-5′thiotriphosphate ([^{35}S]dATPαS) from P2 receptors. Repetitive superfusion with equimolar 2-MeSATP allowed categorization of ARC cells into groups with a high or low (LDD) degree of purinoceptor desensitization, the latter allowing further receptor characterization. Calcium imaging experiments performed at 37 °C vs. room temperature showed further reduction of desensitization. Agonist-mediated intracellular calcium signals were suppressed in all LDD neurons but only 25% of astrocytes in the absence of extracellular calcium, suggestive of metabotropic P2Y receptor expression in the majority of ARC astrocytes. The highly $P2Y_1$-selective receptor agonists MRS2365 and 2-methylthioadenosine-5′diphosphate (2-MeSADP) activated 75–85% of all 2-MeSATP-responsive ARC astrocytes. Taking into consideration the high potency to dose-dependently stimulate ARC cells of the LDD group, the high affinity for rat P2X(1–3) and low affinity for rat P2X4, P2X7 and P2Y receptor subtypes except $P2Y_1$ and $P2Y_{13}$, the agonist 2-MeSATP primarily acted upon P2X2 and $P2Y_1$ purinoceptors to trigger intracellular calcium signaling in ARC neurons and astrocytes.

Key words: hypothalamic arcuate nucleus, primary mixed neuronal and glial microculture, purinoceptor agonist, calcium imaging, radioligand binding, receptor desensitization.

INTRODUCTION

The diencephalic arcuate nucleus (ARC) plays an eminent role in the central control of energy balance, interacting with the parvocellular paraventricular nucleus (pPVN), the ventromedial hypothalamic nucleus (VMH) and the lateral hypothalamic area (LHA) (Chromwall, 1985; Morton et al., 2006; Yi et al., 2006; Krajewski et al., 2010). The ARC is subdivided into a parvocellular dorsomedial (ARCdm) part as well as ventromedial (ARCvm) and -lateral (ARCvl) parts comprised of medium-sized neurons (Simerly, 2015). Afferent neural and enteroendocrine signals from the digestive tract concerning food bolus composition and volume are relayed to these hypothalamic structures *via* the medullary nucleus of the solitary tract (Dockray, 2009; Sam et al., 2012). Humoral factors involved in long-term regulation of energy homeostasis (insulin, leptin and ghrelin), however, can act

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Abbreviations: [^{35}S]dATPαS, [α-^{35}S]deoxyadenosine-5′thiotriphosphate; [Ca^{2+}]i, intracellular calcium concentration; 2-MeSADP, 2-methylthioadenosine-5′diphosphate; 2-MeSATP, 2-methylthioadenosine-5′triphosphate; αβ-meATP, α,β-methyleneadenosine-5′triphosphate; ACTH, adrenocorticotropin; ARC, arcuate nucleus; ATP, adenosine-5′triphosphate; BSA, bovine serum albumin; BT, body core temperature; DA, dopamine; DAPI, 4′,6-diamidino-2-phenylindole; DMH, dorsomedial hypothalamus; EGTA, ethylene glycol tetraacetic acid; GFAP, glial fibrillary acidic protein; HBSS, Hank's Balanced Salt Solution; HDD, high degree of desensitization; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; icv, intracerebroventricular; LDD, low degree of desensitization; LHA, lateral hypothalamic area; MAP2a+b, microtubule-associated protein 2a+b; NPY, neuropeptide Y; NSE, neuron-specific enolase; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PMNGM, primary mixed neuronal and glial microculture; POMC, proopiomelanocortin; pPVN, parvocellular paraventricular nucleus; RLB, radioligand binding buffer; RT, room temperature; TH, tyrosine hydroxylase; VMH, ventromedial hypothalamus.

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directly on ventral ARC cells (Riediger, 2012) due to the presence of fenestrated capillaries, as demonstrated by histo- and physiological approaches in rodents (Shaver et al., 1992; Norsted et al., 2008; Ciofi et al. 2009; Schaeffer et al., 2013). Especially two cell groups of the ARC represent prime targets for these blood-borne signals (Parker and Bloom, 2012): orexigenic ARCvm neurons expressing neuropeptide Y (NPY) and antiorexigenic ARCvl neurons using proopiomelanocortin (POMC) -derived neuropeptides as neurotransmitters (Meister et al., 2006; Colldén et al., 2010). NPYergic neurons are activated by circulating ghrelin released from empty stomach pyloric glands and project to the pPVN to stimulate food intake (Pusztai et al., 2008; Riediger, 2012), whereas they are inhibited by elevated plasma levels of adipose tissue-derived leptin and pancreatic insulin, reflecting a high body fat content as long-term energy store (Crespo et al., 2014). Contrasting actions and effects have been described for all three humoral factors with regard to modulation of POMCergic ARC neurons (Kim et al., 2014).

Only recently, adenosine-5′triphosphate (ATP) got recognized as a potential neurotransmitter involved in the hypothalamic circuitry regulating feeding, based on intracerebroventricular (icv) or local (VMH, LHA) microapplication studies in rats (Kittner et al., 2006; Seidel et al., 2006; Stojilkovic, 2009). Electrophysiological investigations in brain slice preparations (LHA) (Wollmann et al., 2005) and primary cultures of the ARC (Wakamori and Sorimachi, 2004), LHA (Jo and Role, 2002) or VMH (Sorimachi et al., 2001) support the functional expression of neuronal purinoceptors. Binding various purine and/or pyrimidine nucleotides, these can be subdivided into ionotropic P2X and metabotropic P2Y receptor families (Burnstock, 2011). Genes have been identified to code for seven P2X subunits forming both homo- and heterotrimeric receptor proteins, with a channel pore permeable for sodium and calcium ions at diverse gating and desensitization properties (North, 2002; Coddou et al., 2011a). ATP, α,β-methyleneadenosine-5′triphosphate (α,β-meATP) and 2-methylthioadenosine-5′triphosphate (2-MeSATP) are frequently used as agonists to characterize P2X receptor subtype expression (Burnstock, 2007; Jacobson et al., 2006). While α,β-meATP reveals high affinity for P2X(1,3) receptors (Burnstock, 2006), 2-MeSATP binds to all P2X receptor subtypes showing higher affinity for the P2X(1–3,5) as against P2X(4,7) subunits (von Kügelgen, 2008; Coddou et al., 2011b). For the ARC, expression of P2X(2,4,6) but not P2X(1,3) receptors was demonstrated using *in situ* hybridization, with dense labeling of P2X2 receptor-specific mRNA (Collo et al., 1996; Kanjhan et al., 1999). Immunohistochemistry revealed high signals for the P2X2 receptor in neurons and nerve fibers throughout the rat ARCvm (Vulchanova et al., 1996; Xiang et al., 1998; Colldén et al., 2010), whereas both negative and positive results have been reported with regard to P2X5 receptor expression (Collo et al., 1996; Xiang et al., 2006). All eight P2Y receptor subtypes represent G-protein-associated purinoceptors coupled to the intracellular phospholipase C ($P2Y_{1,2,4,6,11}$) or adenylate cyclase ($P2Y_{11–14}$) pathways (Erb et al., 2006). Subsequent modulation of calcium channels in the cell or endoplasmatic reticulum membrane may result in altered intracellular calcium signaling (von Kügelgen, 2006; Jacobson, 2010). Primarily ADP, 2-methylthioadenosine-5′diphosphate (2-MeSADP), and UTP/UDP are employed as agonists to specify functional P2Y receptor expression (Burnstock, 2007; Jacobson et al., 2006). Only $P2Y_1$ and $2PY_{11-13}$ purinoceptors are selective for adenine nucleotides. 2-MeSATP serves as partial agonist of high potency for the $P2Y_1$ receptor (von Kügelgen, 2008), whose expression was described for the rat ARC histochemically (Seidel et al., 2006) and *in vivo* based on modulation of food intake in food-deprived rats by intracranial administration of $P2Y_{1,11}$-selective receptor antagonists (Kittner et al., 2006).

In the present study, we investigate the functional expression of P2 purinoceptors in single neurons and astrocytes of a primary cell culture system derived from microdissected rat ARC tissue. Neuronal expression of ARC-specific transmitters involved in the control of energy homeostasis (NPY, POMC) justifies studies in this cell culture system as being at least partially representative for the *in vivo* situation. Employing P2 purinoceptor agonists in intracellular calcium imaging experiments with repetitive applications, alterations of extracellular calcium concentration and temperature, supports functional expression of P2X2 receptors in ARC neurons and astrocytes as well as $P2Y_1$ receptors in astrocytes, with low desensitization characteristics. Preliminary data obtained with post-experimental neuronal transmitter coding indicate purinergic activation of both POMCergic and NPYergic ARC neurons.

EXPERIMENTAL PROCEDURES

Animals

Male Wistar rat pups obtained from an in-house breeding colony were used to establish a primary mixed neuronal and glial microculture (PMNGM) of the ARC, with parent animals originating from Charles River WIGA (Sulzfeld, Germany). Animal care, breeding and experimental procedures were carried out in accordance with the Hessian ethical committee (GI 18/2-Nr. 66/2011). Animals were housed under controlled conditions at 23 ± 1 °C, relative humidity of 50% and a 12:12-h day: night cycle with artificial lights on from 7 am to 7 pm. The pups were reared in large type IV cages by their mothers, who had access to drinking water and standard chow *ad libitum* (Altromin, Lage-Lippe, Germany).

Isolation and cultivation of ARC-specific PMNGMs

PMNGMs of the rat ARC were established from topographically excised brain tissue of 4–6-day-old rat pups. After decapitation, the brain was quickly removed under aseptic conditions, fixed upright onto a Teflon®-block with Histoacryl® tissue glue (Braun, Melsungen, Germany) and transferred to a chamber filled with ice-cold, oxygenated Gey's Balanced Salt Solution