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Effective G-protein coupling of Y2 receptors along axonal fiber tracts and its relevance for epilepsy

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ABSTRACT

Neuropeptide Y (NPY)-Y2 receptors are G-protein coupled receptors and, upon activation, induce opening of potassium channels or closing of calcium channels. They are generally presynaptically located. Depending on the neuron in which they are expressed they mediate inhibition of release of NPY and of the neuron's classical transmitter GABA, glutamate or noradrenaline, respectively. Here we provide evidence that Y2 receptor binding is inhibited dose-dependently by GTPγS along Schaffer collaterals, the stria terminalis and the fimbria indicating that Y2 receptors are functionally coupled to G-proteins along these fiber tracts. Double immune fluorescence revealed coexistence of Y2-immunoreactivity with β-tubulin, a marker for axons in the stria terminalis, but not with synaptophysin labeling presynaptic terminals, supporting the localization of Y2 receptors along axonal tracts. After kainic acid-induced seizures in rats, GTPγS-induced inhibition of Y2 receptor binding is facilitated in the Schaffer collaterals but not in the stria terminalis. Our data indicate that Y2 receptors are not only located at nerve terminals but also along fiber tracts and are there functionally coupled to G-proteins.

1. Introduction

Neuropeptide Y (NPY) is a member of a family of 36 amino acid peptides that also includes pancreatic polypeptide (PP) and peptide YY (PYY). While PYY and PP are primarily found in the intestine and pancreas, respectively, NPY is widely distributed throughout the peripheral and central nervous systems (Hokfelt et al., 1998). In the brain it is one of the most abundant neuropeptides, mainly contained in subclasses of GABA-ergic and, to a lesser extent, in noradrenergic neurons. Established actions in the central nervous system include its participation in regulation of feeding and drinking and integration of emotions such as anxiety and depression, and NPY may exert endogenous anticonvulsive/neuroprotective functions (Vezzani and Sperk, 2004; Tasan et al., 2016). Among the presently established NPY receptors (Y1, Y2, Y4 and Y5), Y1 and Y2 receptors are most abundant in the brain (Blomqvist and Herzog, 1997). Whereas Y1 receptors are postsynaptic receptors, Y2 receptors are primarily presynaptically located and mediate inhibition of neurotransmitter release (Greber et al., 1994; Klapstein and Colmers, 1997). Both receptors are coupled to G-proteins and may act through activation of potassium channels or inhibition of calcium channels (Ewald et al., 1989). The localization of these receptors can be shown by receptor autoradiography using radiolabeled Leu^{31}, Pro^{34}-PYY, or PYY_{3-36} as Y1- and Y2-selective ligands, respectively (Dumont et al., 1996). To analyze functional coupling of a ligand to its G-protein coupled receptor, activation of GTPγS binding by the receptor ligand (Weiland and Jakobs, 1994; Shaw et al., 2003) as well as, in contrary, modulation of ligand binding by GTPγS have been used (Parker et al., 2007a; Zhen et al., 2015).

Using $[^{125}I]PYY_{3-36}$ receptor autoradiography for Y2 receptors, prominent labeling of the strata radiatum and oriens was observed in sectors CA1 to CA3 of the hippocampus. Immunohistochemistry for Y2 receptors and in situ hybridization for Y2 mRNA containing neurons indicated that this labeling may represent axon fibers of Schaffer collaterals arising from CA3 pyramidal cells (Stanic et al., 2006). Also other fiber tracts, the fimbria and the stria terminalis originating in the hippocampus and bed nucleus of the stria terminalis/amygdala, respectively, are labeled by $[^{125}I]PYY_{3-36}$ (Dumont et al., 1996). This suggests that Y2 receptors may be expressed along these fiber tracts. Whether this labeling represents functionally coupled Y2 receptors or spare receptors, or receptors transported to the respective axon terminal is not known. Interestingly, kainic acid (KA)-induced seizures are followed by a rapid increase in affinity of Y2 receptors in Schaffer collaterals and increased expression in granule cells (Schwarzer et al., 1998). We now aimed to

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investigate whether these receptors located along neuronal fiber tracts are also functionally coupled to G-proteins. We applied Y2 receptor autoradiography using [^{125}I]PYY_{3-36} as radioligand and investigated its inhibition by the non-hydrolizable GTP analogue GTPγS in brain sections from rats exposed to KA-induced seizures and in controls. Inhibition of Y2 agonist binding by GTPγS reflects a shift from a high affinity to a low affinity state and is an index for G-protein coupling (Parker et al., 2007a; Zhen et al., 2015).

2. Methods

2.1. Rats and injection of kainic acid

Adult male Sprague-Dawley rats (210–260 g; Forschungsinstitut für Versuchstierzucht, Himberg, Austria) were used. They had free access to food and water and were housed at a temperature of 22–23 °C, a relative humidity of 50–60% and a 12 h light/dark cycle. All animal experiments were conducted according to national guidelines and European Community laws. They were approved by the *Committee for Animal Protection* of the Austrian Ministry of Science and care was taken to minimize suffering of the rats.

Rats (in total 24) were injected either with 10 mg/kg kainic acid (KA, Ascent Scientific Ltd., North Somerset, United Kingdom, dissolved in 0.9% NaCl at a concentration of 5 mg/ml and adjusted to pH 7.0) or with saline. Seizure behavior was monitored for at least three hours and rated using a 4-stage rating scale described previously (Sperk et al., 1983). Rats with generalized seizures (rating 3 and 4) were killed by exposure to CO_2 gas after 10 days. Brains were rapidly removed from the sculls and snap-frozen in −70 °C isopentane (Merck, Darmstadt, Germany). Brains were cut either coronary or horizontally between the septum and the ventral hippocampus using a cryostat-microtome. Three subsequent sections (20 μm) were always thaw-mounted together on silane-coated slides and then stored at −70 °C. Every 10th slide was subjected to Nissl staining as described (Schwarzer et al., 1998). These sections were used to identify corresponding sections from three rats for receptor autoradiography.

2.2. Receptor autoradiography

For Y2 receptor autoradiography, PYY_{3-36} was radiolabeled with ^{125}I (2200 Ci/mmol; New England Nuclear, DuPont, Boston, USA) using the chloramine T procedure and purified by high performance liquid chromatography (HPLC) as described before (Bellmann et al., 1991). The first radioactive peak presumably containing mono-iodinated Tyr^{36}[^{125}I]PYY was used. Receptor autoradiographies were performed as described before (Dumont et al., 1996; Schwarzer et al., 1998). Slides were thawed and pre-incubated for 30 min at room temperature in 200 ml of Krebs-Henseleit-Tris buffer (118 mM NaCl, 4.8 mM KCl, 1.3 mM $MgSO_4$, 1.2 mM $CaCl_2$, 50 mM glucose, 15 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 10 mM Tris, pH 7.3). The incubation was performed in *Coplin* jars containing 20 ml of the same buffer supplemented with 0.1% bovine serum albumin, 0.05% bacitracin, and 25 pM [^{125}I]PYY_{3-36}. Kinetic analysis of the receptor binding was performed in sections of the dorsal hippocampus of controls and 48 h after KA injection. Incubations were done in triplicates at concentrations of 25 pM [^{125}I]PYY_{3-36}. GTPγS was included at concentrations of 0, 1, 3, 10, 30 and 100 μM. In an initial experiment the dorsal hippocampus was evaluated with the additional concentrations of 0.03, 300 and 1000 μM of GTPγS. Incubations were performed at room temperature for 2 h. Unspecific binding was determined in the presence of 1 μM NPY; it was uniformly distributed throughout the section and was <5% of total binding (e.g. in Schaffer collaterals). The sections were dipped twice and then washed for 30 s in ice-cold Krebs-Henseleit-Tris buffer, dipped in deionized water, and rapidly dried under a stream of cold air. They were then exposed to β-max films for 10 days together with [^{125}I]-microscales (both Amersham, Bucks, U.K.).

2.3. Quantification of receptor autoradiography and statistics

The autoradiographs were developed, digitized through the Appligene Image System (Illkirch, France), and analyzed using the public domain NIH ImageJ program (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–2016). The absorbance was measured in the strata oriens and radiatum of CA1 representing fiber tracts and terminals of the Schaffer collaterals, the septum, piriform cortex, stria terminalis, and the fimbria. Absolute values were calculated by using a dose-response curve of the absorbance obtained through concomitant autoradiography of ^{125}I-microscales and expressed as fmol/mg of wet tissue weight. Specific binding was calculated by subtracting the mean unspecific binding (determined in 35 sections in the presence of 1 μM NPY) from total binding. For studies on the inhibition of [^{125}I]PYY_{3-36} binding by GTPγS, mean values determined in three to six sections per animal were averaged. These values were used for calculating the mean values for 3 to 5 rats. Data are expressed as percentage of total specific [^{125}I]PYY_{3-36} binding. Statistical analysis was done for time course and displacement studies by analysis of variance (ANOVA) and the multiple-comparison Dunnett *posteriori* test.

Values of individual sections were obtained by calculating the mean values of each hemisphere in 3 consecutive sections. Values obtained from 3 to 4 rats were averaged and analyzed. Displacement curves for [^{125}I]PYY_{3-36} binding by GTPγS was evaluated using GraphPad Prism program (GraphPad Prism version 5.00 for Mac OS X, GraphPad Software, San Diego California USA, www.graphpad.com). Mean values from 3 to 4 rats (obtained as mean from 3 consecutive sections) for each GTPγS concentration were obtained and calculated in the GraphPad Prism program (using the non-linear regression (curve fit) and log inhibitor vs. response functions). K_d values for inhibition of [^{125}I]PYY_{3-36} binding by GTPγS were determined individually for each animal. Increase in [^{125}I]PYY_{3-36} binding in KA treated rats vs. controls was determined from the total (without GTPγS) specific binding in the same sections.

2.4. Regression analysis and statistical analyses

Dose-response curves for [^{125}I]PYY_{3-36} binding by GTPγS and statistical analyses were performed using GraphPad Prism. Values of individual sections were obtained by calculating the mean values of each hemisphere in 3 consecutive sections. These values, obtained from 3 to 4 rats, were averaged and analyzed using the non-linear regression (curve fit) and log inhibitor vs. response functions. IC_{50} values for inhibition of [^{125}I]PYY_{3-36} binding by GTPγS were determined individually for each animal and then averaged (±SEM). Increase in [^{125}I]PYY_{3-36} binding in KA treated rats vs. controls was determined from the total (without GTPγS) specific binding in the same sections. For comparison of two groups a two-tailed Student's *t*-test was used. All data are presented as mean ± SEM. Statistical significance was defined as $p < 0.05$.

2.4.1. Immunohistochemistry

Sections from the ventral hippocampus of C57BL/6 mice were used. The brains were perfused with 4% paraformaldehyde in phosphate buffered saline (PBS) and 30 μm sections were cut. For immunohistochemistry, we used Y2 receptor antibody (1:1000, RA14112, Neuromics, Edina, Minnesota, USA through Eubio, Vienna, Austria). It was performed as described previously (Stanic et al., 2006; Wood et al., 2015). As secondary antibody we used a horseradish peroxidase (HRP)-coupled goat anti-rabbit antibody (1:250 P0448; Dako, Vienna, Austria). It was amplified and detected with tyramide fluorescein (TSA fluorescein, signal amplification solution, 1:100, in-house) for fluorescence labeling or directly developed with diamino-benzidine and H_2O_2 (Wood et al., 2015). Sections were mounted on slides and examined in a fluorescence microscope.

Double immunofluorescence was performed for Y2 receptors together with synaptophysin (a marker for synapses) and for Y2 receptors

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