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The effect of bradykinin on the electrical activity of rat myenteric neurons

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ABSTRACT

Q2 Bradykinin is a mediator involved in inflammatory processes in the gut. Here we investigated the effect of bradykinin on the electrical activity of rat myenteric neurons, the key players for regulation of gastrointestinal motility. Bradykinin (2×10^{-8} mol/l) induced a biphasic increase in frequency of action potentials measured with microelectrode arrays. This increase was mirrored by a biphasic increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), which was observed in about 40% of the myenteric neurons. The bradykinin B_1 receptor agonist des-arg^9-bradykinin as well as the bradykinin B_2 receptor agonist hyp^3-bradykinin induced a similar effect on $[Ca^{2+}]_i$. Immunocytochemical stainings confirmed the expression of both receptor types by myenteric ganglionic cells. Real time PCR showed that the inducible B_1 receptor was upregulated during cell culture. The inhibition of cyclooxygenases with piroxicam reduced the effect of bradykinin on the electrical activity of myenteric neurons. The suppression of the glial growth on microelectrode arrays did not affect the bradykinin-induced change in frequency of action potentials. This suggests that prostaglandins, which probably mediate the effect of bradykinin, are not exclusively released from glial cells.

The bradykinin-induced increase in $[Ca^{2+}]_i$ was dependent on the presence of extracellular Ca^{2+} and was inhibited by Co^{2+}, Cd^{2+}, and Ni^{2+}, blockers of voltage-dependent Ca^{2+} channels, indicating a stimulation of the influx of extracellular Ca^{2+} by the kinin. Consequently, bradykinin induces a Ca^{2+} influx in myenteric neurons via Ca^{2+} channels in the plasma membrane.

1. Introduction

Bradykinin is a nonapeptide playing an important role in the mediation of inflammatory processes. During states of injury or inflammation, the precursor protein kininogen is transformed to bradykinin by tissue or plasma kallikrein (Farmer and Burch, 1992; Leeb-Lundberg et al., 2005). In the gut, bradykinin fulfills various functions: it mediates either contraction or relaxation of the intestinal muscle (Wassdal et al., 1999; Würner and Diener, 2013), it leads to chloride secretion – either by direct stimulation of the epithelial cells or indirectly via submucosal secretomotor neurons (Cuthbert and Margolius, 1982; Diener et al., 1988; Baird et al., 2008) – and it induces vasodilatation and increases the permeability of blood vessels (Regoli and Barabe, 1980; Farmer and Burch, 1992). In addition, the kinin is involved in the development of gastrointestinal pain by stimulation of serosal afferent neurons (Maubach and Grundy, 1999).

Bradykinin binds to two different receptors: the bradykinin B_1 receptor and the bradykinin B_2 receptor (for review see Prado et al., 2002; Leeb-Lundberg et al., 2005). Whereas the bradykinin B_2 receptor is constitutively expressed in various tissues, the bradykinin B_1 receptor is induced during tissue injury and inflammation and can be found only in tiny amounts under healthy conditions. Usually, both receptors are G_q protein-coupled receptors activating phospholipase C and thus leading to the formation of inositol-1,4,5-trisphosphate (IP_3), which stimulates the release of Ca^{2+} from the endoplasmic reticulum (Gelperin et al., 1994). However, a bradykinin-induced influx of Ca^{2+} through voltage-dependent Ca^{2+} channels is observed in the rat submucosal plexus as well (Avemary and Diener, 2010).

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It is known from many tissues that bradykinin can stimulate a release of prostaglandins (Baird et al., 2008; Liu et al., 2012). In the rat myenteric plexus there is evidence that bradykinin is able to stimulate neurons via prostaglandins (Gelperin et al., 1994), which are assumed to be derived from glial cells (Murakami et al., 2007). Similarly, the excitation of submucosal neurons induced by bradykinin is mediated by prostaglandins in guinea pig (Hu et al., 2004b), whereas in rat another class of metabolites of arachidonic acid, i.e. leukotrienes, is responsible for the effect of the kinin (Avemary and Diener, 2010).

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The present study aimed to investigate the effects of bradykinin on the electrical activity of rat myenteric neurons. The myenteric plexus is a nerve plexus located between the longitudinal and the circular muscle layer and is part of the enteric nervous system. It is mostly involved in the regulation of muscle activity such as the peristaltic reflex (Furness, 2006). This plexus consists of sensory neurons, interneurons and motor neurons, which build a reflex circuitry enabling the myenteric plexus to alter motoric activity independently from the central nervous system (Schemann and Neunlist, 2004; Furness, 2006). A stimulation of myenteric neurons by bradykinin might therefore lead to changes in the motility of the gut, possibly causing diarrhea. Since bradykinin is an inflammatory mediator, it is not surprising that this peptide is also involved in inflammatory bowel diseases. In patients suffering from these diseases, the bradykinin B_1 receptor was found to be upregulated in enterocytes (Stadnicki et al., 2005). The release of the bradykinin forming enzyme kallikrein was increased as well (Devani et al., 2005).

We therefore aimed to investigate how bradykinin alters neuronal activity of rat myenteric neurons. Since there seems to be a great variety among different species, we also intended to clarify the mechanisms and signal transduction involved in this effect. For this purpose microelectrode arrays and Ca^{2+} imaging were used. Furthermore, using immunofluorescence analysis and real time PCR we aimed to find out where both bradykinin receptors are localized and whether an in vitro upregulation of the bradykinin B_1 receptor can be induced.

2. Material and methods

2.1. Animals

The experiments were carried out with Wistar rats bred in the Institute for Veterinary Physiology and Biochemistry at Justus Liebig University in Giessen, Germany. They were housed in small groups at a constant room temperature of 22.5 °C, air humidity of 50–55% and 12 h:12 h light:dark cycle. The animals were provided with standard rat diet and water ad libitum. Myenteric ganglia were prepared from male and female animals with an age of five to nine days. The animals were killed by decapitation (approved by Regierungspräsidium Giessen, Giessen, Germany).

2.2. Solutions

The preparation of myenteric ganglia was carried out in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (HBSS; Life Technologies, Darmstadt, Germany) containing 20 mmol/l 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), penicillin (10.000 units/ml) and streptomycin (10 mg/ml) (Biochrom, Berlin, Germany). Cell culture was performed in Neurobasal A medium (Life Technologies) complemented with 10% (v/v) fetal calve serum (PAA, Colbe, Germany), penicillin/streptomycin (in the same concentration as above) and 0.5 mmol/l glutamine (Sigma-Aldrich, Taufkirchen, Germany). The microelectrode array measurements were performed in Ca^{2+}- and Mg^{2+}-containing HBSS, supplemented with HEPES and penicillin/streptomycin in the same concentrations as described above.

In the imaging experiments cells were superfused with a standard Tyrode solution consisting of (in mmol/l) 140 NaCl, 5.4 KCl, 10 HEPES, 1 $CaCl_2$, 1 $MgCl_2$, and 12.2 glucose. The pH was set at 7.4 with HCl/NaOH. The Ca^{2+}-free Tyrode solution consisted of (in mmol/l): 140 NaCl, 5.4 KCl, 10 HEPES, 1 $MgCl_2$, and 12.2 glucose with a pH set to 7.4 using NaOH/HCl. For immunocytochemistry a phosphate-buffered saline (PBS) was used containing (in mmol/l) 10 sodium phosphate buffer, 120 NaCl and 2.7 KCl with an pH of 7.4.

2.3. Preparation of myenteric ganglionic cells

The whole intestine was removed and placed into Ca^{2+}- and Mg^{2+}-free HBSS. While the large intestine was discarded, the small intestine was placed under a binocular microscope and the muscle layer was stripped off using two fine forceps. The muscle layer was incubated in a collagenase type II solution (0.5 mg/ml; Biochrom) at 37 °C for 80 min.

With the use of a binocular microscope, the net-like ganglionic cells were collected using a micropipette and placed into warm Neurobasal medium. Depending on further use, 10 µl of the cell suspension was seeded either on coverslips coated with poly-L-lysin (5 µg/ml) or microelectrode arrays coated with poly-L-lysin (5 µg/ml) and laminin (1 mg/ml) (Medert et al., 2013). After 1 h, the cells had settled down, were covered with 1 ml neurobasal medium and incubated at 37 °C in an atmosphere of 5% (v/v) CO_2 in 95% (v/v) air for at least 16 h. For the suppression of glial growth some cells were cultured in the presence of 10^{-5} mol/l cytosine arabinoside.

After termination of the electrical recordings (see below), the microelectrode arrays were cleaned by incubation in an enzymatic solution (Tergazyme; Sigma-Aldrich) overnight at room temperature.

2.4. Microelectrode arrays

The microelectrode array measurements were performed with a USB-MEA A60 system from Multi Channel Systems (Reutlingen, Germany). The arrays were provided with 60 electrodes fabricated of titanium nitride with a diameter of 30 µm and an electrode spacing of 200 µm. They were grounded by an internal reference electrode. The raw data was sampled at 10 kHz and low-pass filtered at a frequency of 20 Hz. The data was collected and analyzed with the software MCRack (Multi Channel Systems).

After the ganglionic cells were cultured for one to three days, the measurements were performed in HBSS on a heat plate set to 37 °C. Each microelectrode array was used one to three times per day for administration of different substances with at least 2 h recovery time between the measurements. Test substances were administered using a micropipette.

The spike rate was measured by counting those spikes passing a certain threshold. Due to differences among the microelectrode arrays, the threshold was set individually for each experiment. Depending if the electrode showed a negative or a positive spiking, the threshold was set 10 µV underneath or above the lowest/highest value of noise. The baseline was measured just prior to the administration of the substance. In order to quantify the biphasic response induced by bradykinin, the spike rate was determined in 24 consecutive 30 s intervals. Peak 1 was defined as the maximal activity reached within 30–90 s and peak 2 as the maximum reached $\geq$ 120 s after administration of the kinin.

In order to distinguish signals from different neurons measured by the same electrode, a waveform analysis was performed using the spike sorting function of MCRack. One electrode is able to measure several cells in its surrounding area, with each cell having another distance from the electrode. This difference in distance is reflected by different spike waveforms. With the waveform analysis up to three different units per electrode could be separated from each other. The percentage of responding neurons in relation to all active neurons was calculated. All neurons showing an increase in frequency of action potentials higher than the absolute value of 0.3 Hz in response to bradykinin were defined as responder.

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