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Recordings from human myenteric neurons using voltage-sensitive dyes

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ABSTRACT

Voltage-sensitive dye (VSD) imaging became a powerful tool to detect neural activity in the enteric nervous system, including its routine use in submucous neurons in freshly dissected human tissue. However, VSD imaging of human myenteric neurons remained a challenge because of limited visibility of the ganglia and dye accessibility. We describe a protocol to apply VSD for recordings of human myenteric neurons in freshly dissected tissue and myenteric neurons in primary cultures. VSD imaging of guinea-pig myenteric neurons was used for reference. Electrical stimulation of interganglionic fiber tracts and exogenous application of nicotine or elevated KCl solution was used to evoke action potentials. Bath application of the VSDs Annine-6Plus, Di-4-ANEPPS, Di-8-ANEPPQ, Di-4-ANEPPDHQ or Di-8-ANEPPS revealed no neural signals in human tissue although most of these VSD worked in guinea-pig tissue. Unlike methylene blue and FM1–43, 4-Di-2-ASP did not influence spike discharge and was used in human tissue to visualize myenteric ganglia as a prerequisite for targeted intraganglionic VSD application. Of all VSDs, only intraganglionic injection of Di-8-ANEPPS by a volume controlled injector revealed neuronal signals in human tissue. Signal-to-noise ratio increased by addition of dipicrylamine to Di-8-ANEPPS (0.98 ± 0.16 vs. 2.4 ± 0.62). Establishing VSD imaging in primary cultures of human myenteric neurons led to a further improvement of signal-to-noise ratio. This allowed us to routinely record spike discharge after nicotine application. The described protocol enabled reliable VSD recordings from human myenteric neurons but may also be relevant for the use of other fluorescent dyes in human tissues.

1. Introduction

The enteric nervous system (ENS) primarily consists of two ganglionated plexi which are embedded in the gut wall. The ENS acts as an autonomous nervous system which controls the main gut functions. This is achieved by the submucous plexus which mainly regulates mucosal functions while the myenteric plexus, located between the two muscle layers, coordinates smooth muscle activity.

Electrophysiological properties of myenteric neurons have been studied with conventional microelectrode techniques, mainly in small laboratory animals (Wood, 1970, 1973; Ohkawa and Prosser, 1972; Nishi and North, 1973; Hirst et al., 1974; Furukawa et al., 1986; Brookes et al., 1988; Browning and Lees, 1996; Cornelissen et al., 2001). Such studies have advanced our knowledge on basic functions of the ENS in guinea-pigs, cats, mice, rats and pig, but did also highlight species-specific ENS functions. There has been much less progress in the neurobiology of the human ENS as the basis to understand pathophysiology of functional, structural and inflammatory gastrointestinal diseases. This is mainly due to technical obstacles preventing the routine application of conventional microelectrode techniques to human intestinal tissue. In pioneering studies Obaid et al. (1992, 1999) and Neunlist et al. (1999), adapted multi-site optical imaging systems in combination with a voltage-sensitive dye (VSD) to record from enteric neurons in the guinea-pig. With this technique, we were able to also routinely record from human submucous neurons with a high spatial and temporal resolution that revealed fast changes in membrane potential such as action potentials and fast excitatory postsynaptic potentials (EPSPs) (Schemann et al., 2002, 2005; Michel et al., 2005; Breunig et al., 2007; Buhner et al., 2009).

However, recordings from the human myenteric ganglia remained a challenge because of their limited visibility and dye accessibility. So far only two studies on the electrophysiology of human myenteric neurons have been published. They used conventional intracellular recordings to investigate basic properties of cultured myenteric neurons (Maruyama, 1981) or myenteric neu-

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rons from freshly dissected human colon (Brookes et al., 1987). Both studies had limitations: the number of studied neurons was rather small with 2 and 43, respectively. Many years required to record from such small number of neurons are one explanation why such studies have not been followed up. In addition, the study by Maruyama used cultured fetal myenteric neurons which very likely do not reflect behaviour of mature enteric neurons.

We have already demonstrated that in principle it is feasible to record from human myenteric neurons using voltage-sensitive dye imaging (Schemann et al., 2002). However, we were only successful in one tissue and reliable recordings of reproducible signals from human myenteric neurons remained a challenge. Therefore, it was the aim of our study to establish a protocol that allows the successful use of VSD imaging for recordings of human myenteric neurons. The protocols were tested in freshly dissected human tissues as well as in primary cultures of human myenteric neurons. We investigated the usefulness of several voltage-sensitive dyes both in human as well as in guinea-pig myenteric plexus. As visibility of intact, non-cultured, human myenteric ganglia is a limitation we used several vital dyes to pre-stain the ganglia. Myenteric plexus preparations from guinea-pigs were used to test whether these dyes alter electrophysiological behaviour of myenteric neurons which would exclude their use in human tissue.

2. Materials and methods

2.1. Guinea-pig myenteric plexus preparations

For the experiments we used male Dunkin Hartley guinea-pigs (Charles River Laboratories, Kisslegg, Germany; Harlan GmbH, Borchen, Germany). After killing the animals by cervical dislocation followed by exsanguination, the ileum was quickly removed and dissected in ice cold Krebs solution to obtain longitudinal muscle myenteric plexus preparations. The procedures were according to the German animal protection law. During preparation the tissue was constantly perfused with ice cold Carbogen (5% CO_2, 95% O_2) gassed Krebs solution (pH 7.4) containing (in mM): 117 NaCl, 4.7 KCl, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, 2.5 $CaCl_2$ and 11 glucose. The preparation was placed in a recording chamber and continuously perfused with 37 °C Krebs solution (pH 7.4) containing (in mM): 117 NaCl, 4.7 KCl, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 20 $NaHCO_3$, 2.5 $CaCl_2$ and 11 glucose. All chemicals were obtained from Sigma–Aldrich (Schnelldorf, Germany).

2.1.1. Screening vital dyes to visualize human myenteric ganglia

To check if the dyes used for pre-staining human myenteric ganglia had any unspecific effects on the electrophysiological behaviour, we performed the following tests in freshly dissected guinea-pig longitudinal muscle myenteric plexus preparations. After staining the ganglia with 20 μM of the voltage-sensitive dye Di-8-ANEPPS, we evoked control fast EPSPs and compound action potentials by electrical stimulation of interganglionic fiber tracts with supramaximal stimulus strength using a Teflon-coated platinum electrode with a diameter of 25 μm (101R-1T; Science Products GmbH, Hofheim, Germany). The tissues were then incubated with the vital dyes methylene blue (50 μM for 15 min), FM1–43 (4 μM for 10 min) which labels nerve terminals during synaptic activity (Betz and Bewick, 1992) or 4-Di-2-ASP (2.5 μM for 10 min) which is a cationic mitochondrial dye staining nerve terminals (Magrassi et al., 1987). After the incubation we performed another electrical fiber tract stimulation to investigate changes in compound action potentials and fast EPSPs.

2.1.2. Screening different voltage-sensitive dyes to record from human myenteric ganglia

In our previous experiments in guinea-pig and human submucous neurons we exclusively used the voltage-sensitive dye Di-8-ANEPPS (Neunlist et al., 1999; Schemann et al., 2002). As it is known that membrane properties of different cell types determine the suitability of voltage-sensitive dyes (Cohen and Salzberg, 1978; Waggoner, 1979; Fluhler et al., 1985; Loew et al., 1992; Loew, 1996; Wu et al., 1998; Zochowski et al., 2000) we tested several voltage-sensitive dyes for their use in human myenteric neurons. All these dyes were first tested in guinea-pig myenteric plexus preparations to check tissue dye accessibility and their suitability to record action potentials.

In guinea-pig preparations we used 2.5–50 μM ANNINE-6Plus which is a water soluble voltage-sensitive dye displaying strong binding to lipid membranes (Kuhn and Fromherz, 2003; Fromherz et al., 2008). We used Di-4-ANEPPDHQ which is less phototoxic, more water soluble and leads to larger fluorescence signals than Di-8-ANEPPS (Obaid et al., 2004) at concentrations of 7.5–75 μM. Di-4-ANEPPS, which incorporates into the cell membrane faster than Di-8-ANEPPS (Fluhler et al., 1985; Fromherz and Lambacher, 1991; Bedlack et al., 1992) was used at 10 μM. Another analogue of Di-8-ANEPPS is the more hydrophobic dye Di-8-ANEPPQ (Tsau et al., 1996) which was used at a concentration of 20 μM. ANNINE-6Plus and Di-4-ANEPPDHQ were tested in addition with and without Pluronic F-127 (Invitrogen, Karlsruhe, Germany). To increase the signal-to-noise ratio for experiments in freshly dissected human myenteric plexus preparations we used DPA (dipicrylamine) as a resonance-energy-transfer acceptor as described elsewhere (Bradley et al., 2009; Sjulson and Miesenböck, 2008; Chanda et al., 2005a,b). DPA is a non-fluorescent hydrophobic anion, which localizes at the lipid–aqueous interface and can be used as a classical Förster-energy-transfer acceptor from variety of donor fluorophores (Chanda et al., 2005a).

All voltage-sensitive dyes were purchased from Invitrogen except methylene blue (Sigma–Aldrich). ANNINE-6plus was generously supplied by Prof. Dr. Peter Fromherz (Department of Membrane and Neurophysics; Max Planck Institute, Munich).

2.2. Human myenteric plexus preparations

The studies on human myenteric neurons were performed using surgical specimens from patients undergoing abdominal surgery in the Departments of Surgery at the Medical Clinic in Freising and at the Medical Clinic of the Technische Universität München. Samples were taken from macroscopically normal, unaffected areas as determined by visual inspection of the pathologist. The protocol was approved by the ethic committee of the Technische Universität München (project approval: 1746/07).

2.2.1. Freshly dissected human tissue

After removal, the surgical specimens were placed in ice cold, oxygenated, sterile Krebs solution and immediately transferred to the laboratory. We isolated the myenteric plexus by gently removing the mucosa, submucosa and the circular and longitudinal muscle layer. During the dissection the tissue was constantly perfused with ice cold Carbogen gassed Krebs solution. The myenteric plexus preparation was then pinned onto a silicone ring (Down Cornig, Midland, TX, USA), placed in the recording chamber and continuously perfused with 37 °C Krebs solution.

To visualize the ganglia, we first incubated the tissue for 10–15 min with 4-Di-2-ASP (2.5 μM) followed by 5–15 min wash out time in order to visualize the ganglia.

For human myenteric plexus we used three staining protocols to label ganglia with voltage-sensitive dyes. The first approach was to stain individual ganglia by local pressure application of the dye