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L-type calcium channels contribute to 5-HT_3-receptor-evoked CaMKIIα and ERK activation and induction of emesis in the least shrew (*Cryptotis parva*)

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ABSTRACT

Activation of serotonergic 5-HT_3 receptors by its selective agonist 2-methyl serotonin (2-Me-5-HT) induces vomiting, which is sensitive to selective antagonists of both 5-HT_3 receptors (palonosetron) and L-type calcium channels (LTCC) (amlodipine or nifedipine). Previously we demonstrated that 5-HT_3 receptor activation also causes increases in a palonosetron-sensitive manner in: i) intracellular Ca^{2+} concentration, ii) attachment of calmodulin (CaM) to 5-HT_3 receptor, and iii) phosphorylation of Ca^{2+}/calmodulin-dependent protein kinase IIα (CaMKIIα) and extracellular-signal-regulated kinase 1/2 (ERK1/2). Here, we investigate the role of the short-acting LTCC blocker nifedipine on 2-Me-5-HT-evoked intracellular Ca^{2+} increase and on downstream intracellular emetic signaling, which have been shown to be coupled with 2-Me-5-HT's emetic effects in the least shrew.

Using the cell-permeant Ca^{2+} indicator fluo-4 AM, here we present evidence for the contribution of Ca^{2+} influx through LTCCs (sensitive to nifedipine) in 2-Me-5-HT (1 μM) -evoked rise in cytosolic Ca^{2+} levels in least shrew brainstem slices. Nifedipine pretreatment (10 mg/kg, s.c.) also suppressed 2-Me-5-HT-evoked interaction of 5-HT_3 receptors with CaM as well as phosphorylation of CaMKIIα and ERK1/2 in the least shrew brainstem, and 5-HT_3 receptors -CaM colocalization in jejunum of the small intestine. In vitro exposure of isolated enterochromaffin cells of the small intestine to 2-Me-5-HT (1 μM) caused CaMKIIα phosphorylation, which was also abrogated by nifedipine pretreatment (0.1 μM). In addition, pretreatment with the CaMKII inhibitor KN62 (10 mg/kg, i.p.) suppressed emesis and also the activation of CaMKIIα, and ERK in brainstem caused by 2-Me-5-HT (5 mg/kg, i.p.). This study provides further mechanistic explanation for our published findings that nifedipine can dose-dependently protect shrews from 2-Me-5-HT-induced vomiting.

1. Introduction

Chemotherapy-induced nausea and vomiting (CINV) is mediated via interactions of neuronal and neurochemical circuits involving the enteric nervous system (ENS), the vagus and enterochromaffin cells (EC cells) in the gastrointestinal tract (GIT), and the medullary emetic nuclei of the dorsal vagal complex (DVC) in the brainstem (Darmani et al., 2009; Minami et al., 2003a). The medullary DVC includes the nucleus tractus solitarius (NTS), the dorsal motor nucleus of the vagus (DMNX) and the area postrema (AP) (Darmani et al., 2009; Darmani and Ray, 2009). Serotonin (5-hydroxytryptamine=5-HT) is one key emetic neurotransmitter in both the DVC and GIT that contributes to the induction of both the early and delayed phases of CINV (Darmani et al., 2009) via activation of its ligand-gated ion channel, the 5-HT_3 receptor (Darmani and Ray, 2009; Johnston et al., 2014). High levels of 5-HT_3 receptors are found in the brainstem, especially in areas involved in the initiation and coordination of the vomiting reflex such as AP, NTS, and DMNX (Darmani and Ray, 2009; Hannon and Hoyer, 2008). In addition, large numbers of 5-HT_3 receptors are

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found in the peripheral emetic loci including the ENS, vagal afferents in the GIT, and enterochromaffin (EC) cells (Darmani and Ray, 2009; Machu et al., 1999).

5-HT is a nonselective serotonin receptor agonist and cannot cross the blood brain barrier. Although 5-HT does not cause vomiting in ferrets when administered systemically (Johnston et al., 2014), it does so in the least (*Cryptotis parva*) and house musk (*Suncus murinus*) shrews (Darmani, 1998; Torii et al., 1991). On the other hand, its brain penetrable analog 2-Me-5-HT is not only a more-selective 5-HT_3 receptor agonist, it also causes profound vomiting in all of the above discussed species (Darmani, 1998; Darmani and Johnson, 2004; Ray et al., 2009; Johnston et al., 2014). Unlike the large laboratory emesis models (cats, dogs, or ferrets), shrews are smaller and are considered to be closer to primates than rodents, lagomorphs and carnivores (Churchfield, 1990). The adult house musk shrew is similar in size to an adult rat, whereas the least shrew is relatively smaller (adult weighing 4–6 g) and appears to be easier and cheaper for screening the vomiting and anti-vomiting effects of drugs. Moreover, house musk shrew does not vomit in response to the dopamine D_2 receptor apomorphine (Ueno et al., 1987), and is a relatively less sensitive model to the emetic effects of cisplatin (Sam et al., 2003).

The DVC and the GIT are important sites for the emetic activity of 2-Me-5-HT following its systemic administration (Darmani and Johnson, 2004; Ray et al., 2009). Recently we utilized pharmacological, behavioral, immunohistochemical, and Western blot methods to reveal the central and peripheral emetic signaling components downstream of 5-HT_3 receptor activation in the process of 2-Me-5-HT-evoked vomiting (Zhong et al., 2014b). Administration of 2-Me-5-HT significantly: i) increased intracellular Ca^{2+} level in least shrew brainstem slices, ii) enhanced the interaction of 5-HT_3 receptor with calmodulin (CaM) in the brainstem, as well as their colocalization in AP of brainstem and jejunum of small intestine; and iii) caused sequential phosphorylation of Ca^{2+}/calmodulin-dependent protein kinase IIα (CaMKIIα) and extracellular-signal-regulated kinase 1/2 (ERK1/2) in the brainstem. In vitro exposure of isolated EC cells to 2-Me-5-HT also led to CaMKIIα activation. All these effects were suppressed by pretreatment with the selective 5-HT_3 receptor antagonist, palonosetron. The long-acting LTCCs blocker amlodipine also reversed the above effects following 2-Me-5-HT treatment.

Significant differences in efficacy exist between the short- (nifedipine) and long-acting (amlodipine) LTCC antagonists against some emetogens, including 2-Me-5-HT (Darmani et al., 2014; Zhong et al., 2014a). The role of LTCC in 2-Me-5-HT-evoked post 5-HT_3 receptor signaling requires further elucidation. Thus, in the present study, we determined the inhibitory potential of the short-acting LTCC blocker nifedipine on 2-Me-5-HT-evoked increase in cytoplasmic Ca^{2+} level in brainstem slices. Subsequently, we investigated the inhibitory effects of nifedipine on 5-HT_3 receptor-CaM colocalization and activation of CaMKIIα as well as ERK1/2 caused by systemic administration of 2-Me-5-HT (5 mg/kg, i.p.). In addition, we assessed the effect of nifedipine on CaMKIIα activation in isolated EC cells when exposed in vitro to 2-Me-5-HT.

2. Material and methods

2.1. Animals

Adult least shrews were bred in the animal facility of the Western University of Health Sciences. Previous studies had demonstrated no gender differences, so both males and females were used. Shrews were housed in groups of 5–10 on a 14:10 dark cycle, fed with food and water ad libitum as described previously (Darmani et al., 1999). All the shrews used were 45–60 days old and weighed between 4–5 g. All animals received care according to the Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services Publication, revised, 1985). All experiments were conducted in accordance with Western University IACUC standards.

2.2. Drugs

2-Methyl serotonin maleate salt (2-Me-5-HT) was purchased from Sigma/RBI (St. Louis, MO) and was dissolved in water. Nifedipine was obtained from Sigma/RBI (St. Louis, MO) and KN62 from Calbiochem (San Diego, CA) and both were dissolved in 25% DMSO in water. All drugs were administered at a volume of 0.1 ml/10 g of body weight.

2.3. Ca^{2+} imaging

Transverse slices of least shrew brainstem (200 μm-thick) containing the DVC emetic nuclei were identified and prepared as described in our recent publication (Zhong et al., 2014b). Since only one section (200 μm-thick) containing the emetic nuclei could be collected from each shrew brainstem, slices from 4 different shrews were used to investigate the effect of nifedipine on 2-Me-5-HT-elicited Ca^{2+} increase in the AP region among the various brainstem DVC emetic nuclei. Brainstem slices were incubated with the cell permeant Ca^{2+} indicator, fluo-4 AM (5 μM; Invitrogen) for 30 min in dark at room temperature. After washing by perfusion with oxygenated artificial cerebrospinal fluid (aCSF), slices were pretreated with the LTCC blocker, nifedipine (0.1 μM) or its vehicle for 30 min. During the pretreatment, slices were simultaneously placed in an open bath imaging chamber (Warner Instruments, Hamden, CT) containing aCSF and mounted on the confocal imaging stage assembled with model 710 NLO (Carl Zeiss Microscopy,Thornwood, NY) laser scanning confocal imaging workstation with an inverted microscope (Olympus IX81 or Zeiss Axio Observer Z1). 2-Me-5-HT (1 μM) was added to aCSF containing nifedipine or vehicle at the end of pretreatment using a hand pipette, exactly at the 400 s during the whole 1200-s Ca^{2+} image-acquisition period. Ca^{2+} spark recordings were made using Zeiss C-Apochromat 63 × /1.20 water immersion objective. Calcium image acquisition within the region of interest, the AP region of the brainstem, and data analysis by NIH-approved Fiji ImageJ software, using the time series analyzer plugin for ImageJ, were performed as described in our recent report (Zhong et al., 2014b). Regions of interest (ROI) were selected from the initial frame captured at 0 s, with cells showing initial fluorescence intensities between 5000–25,000, and the values of fluorescence intensities at different time points were identified by time series analyzer to plot the graphs of selected ROI. To show the changes in Ca^{2+} levels before and after 2-Me-5-HT treatment, the average fluorescence intensities were calculated for at least 12 regions of interest in each data acquisition set for all time points. The data is represented in a graph as the ratio (F/F_0) of final fluorescence intensity (F) for each time point to the initial fluorescence intensities (F_0) at 0 s for ROIs and is the mean value of 4 individual animals.

2.4. Behavioral studies of emesis

On the day of the experiment shrews were brought from the animal facility, separated into individual cages and allowed to adapt for at least 2 h. 2 h before the experiment daily food was withheld but shrews were given 4 mealworms each prior to injection, to aid in identifying wet vomits as described before (Darmani, 1998). We have previously demonstrated that a 5 mg/kg intraperitoneal (i.p.) injection of 2-Me-5-HT produces a robust frequency of vomiting in all tested animals (Darmani and Johnson,