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A RAT KNOCKOUT MODEL IMPLICATES TRPC4 IN VISCERAL PAIN SENSATION

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Abstract—Acute and chronic pain resulting from injury, surgery, or disease afflicts >100 million Americans each year, having a severe impact on mood, mental health, and quality of life. The lack of structural and functional information for most ion channels, many of which play key roles in the detection and transmission of noxious stimuli, means that there remain unidentified therapeutic targets for pain management. This study focuses on the transient receptor potential canonical subfamily 4 (TRPC4) ion channel, which is involved in the tissue-specific and stimulus-dependent regulation of intracellular Ca^{2+} signaling. Rats with a transposon-mediated TRPC4-knockout mutation displayed tolerance to visceral pain induced by colonic mustard oil (MO) exposure, but not somatic or neuropathic pain stimuli. Moreover, wild-type rats treated with a selective TRPC4 antagonist (ML-204) prior to MO exposure mimicked the behavioral responses observed in TRPC4-knockout rats. Significantly, ML-204 inhibited visceral pain-related behavior in a dose-dependent manner without noticeable adverse effects. These data provide evidence that TRPC4 is required for detection and/or transmission of colonic MO visceral pain sensation. In the future, inhibitors of TRPC4 signaling may provide a highly promising path for the development of first-in-class therapeutics for this visceral pain, which may have fewer side effects and less addictive potential than opioid derivatives.

Key words: transposon, gene knockout, gastrointestinal tract, somatic pain, morphine alternatives, colon.

INTRODUCTION

Visceral pain is associated with various acute and chronic disease states and does not respond adequately to current pain therapeutics. Visceral pain is often caused by distension, obstruction, or inflammation of the gastrointestinal tract. Nervous pathways involved in visceral pain transmission include the peripheral sensory fibers in the intestinal wall that pass through sympathetic chain ganglia to their spinal ganglia cell bodies, which then innervate neurons located in the layers I, II, V and X of the spinal cord (Ness and Gebhart, 1990). The elucidation of the molecular basis of pain is progressing and promises to deliver novel targets for the development of effective pain therapeutics as alternatives to morphine.

This study focuses on the role of the TRPC4 gene in a rat model of visceral pain induced by intra-colonic administration of mustard oil (MO). The TRPC4 channel, involved in the tissue-specific and stimulus-dependent regulation of intracellular Ca^{2+} signaling, belongs to a superfamily of plasma membrane transient receptor potential (TRP) channels, which are divided into seven subfamilies (Nilius et al., 2007). The TRP canonical subfamily (TRPC) family includes seven structurally related orthologs, TRPC1 to TRPC7 (Henley and Poo, 2004; Gomez and Zheng, 2006). TRP channels operate either as primary detectors of chemical and physical stimuli, as secondary transducers of ionotropic or metabotropic receptors, or as ion transport channels. Both TRPC4 expression and function have been documented in the brain (Mori et al., 1998; Riccio et al., 2002; Fowler et al., 2007). TRPC4 is also present in peripheral sensory neurons (Wu et al., 2008) as well as throughout the gastrointestinal tissue. TRPC4 mRNA and immunoreactivity was shown to be present in nerves innervating both the circular and the longitudinal muscles arising from the muscle-myenteric plexus, submucosal plexus and myenteric ganglia (Liu et al., 2008). Several TRP superfamily members play an important part in the control of GI motility and visceral sensation (Boesmans et al., 2011). Like other TRPCs,

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Abbreviations: ANOVA, analysis of variance; DRG, dorsal root ganglia; ECG, electrocardiogram; EDTA, ethylenediaminetetraacetic acid; ISH, *in situ* hybridization; KO, knockout; MO, mustard oil; PCR, polymerase chain reaction; SSC, saline-sodium citrate; TRP, transient receptor potential; TRPC, TRP canonical subfamily; WT, wild type.

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TRPC4 is postulated to play a role in the functional neurobiology of the enteric nervous system, including calcium homeostasis, membrane excitability, synaptic transmission and axon guidance. However, its role in sensory function, whether somatosensory or viscerosensory, including pain, has not been studied *in vivo* but will be addressed here.

In this study, behavioral tests and *in situ* hybridization (ISH) assays were performed to explore the role of TRPC4 in peripheral somatosensory and viscerosensory pain pathways. We utilized a novel transposon-mediated TRPC4 knockout (KO) model and wild-type (WT) controls to examine the behavioral consequences of noxious stimulation with intracolonic MO. Data show that TRPC4 KO rats do not display the typical MO-induced effects seen in WT rats. Lastly, consistent with the notion that TRPC4 plays a key role in MO-induced pain behaviors, WT rats treated with ML-204, a selective TRPC4 channel antagonist (Miller et al., 2011), also displayed resistance to the noxious effects of intracolonic MO. Data presented in this study provide strong evidence that TRPC4 plays an essential role in the transmission of MO-induced visceral pain.

EXPERIMENTAL PROCEDURES

All procedures were consistent with the guidelines for Ethical Treatment of Research Animals published by the International Association for the Study of Pain and the National Institutes of Health Guide for Use of Experimental Animals to minimize animal use and discomfort. Procedures were approved by the Animal Care and Use Committee at the University of Kentucky. Animals received food and water *ad libitum* and were kept on a 12-h day–night cycle. Animals were raised and handled from birth by laboratory staff to facilitate acclimation to von Frey testing in order to minimize variability between animals within the experimental groups (outlined below).

Generation of TRPC4 KO rats

Insertional mutant rats F344- *TRPC4*$^{Tn(sb\text{-}T2/Bart3)2.192Mcwi}$ (heterozygous, *TRPC4*$^{+/-}$) were provided as "seed rats" by Transposagen Biopharmacueticals Inc., Lexington, Kentucky, United States (www.transposagenbio.com). The KO rats were generated by random insertional gene trapping using the Sleeping Beauty transposon vector (T2/Bart3), which contains transcriptional termination elements (by Medical College of Wisconsin) (Lu et al., 2007). The transposon integrated within intron 1 of TRPC4 and resulted in the ablation of all isoforms containing exon 1. *TRPC4*$^{-/-}$ rats (250–400 g) were bred in Dr. Westlund's laboratory at the the University of Kentucky. The *TRPC4*$^{-/-}$ rats were viable and bred equally well compared to inbred Fisher 344 WT rats. Littermate *TRPC4*$^{-/-}$, *TRPC4*$^{+/-}$, WT male and female rats were used in experimental and control groups. Since the *TRPC4*$^{-/-}$ mutation is in the inbred Fisher 344 line, commercially available Fisher 344 rats represent appropriate WT controls for the studies.

Polymerase chain reaction (PCR) genotyping

All rats bred at the University of Kentucky were genotyped by PCR. The genotype of each animal was confirmed using three primers designed by Transposagen to test WT, *TRPC4*$^{+/-}$ and *TRPC4*$^{-/-}$ rat pups:

5′-GTGTTGGTCTCCATTACTTCAGCT-3′, 5′-ATTCTTCCCTTTGAGCCCACT-3′, and transposon primer 5′-CTGACCTAAGACAGGGAATT-3′. PCR was carried out at 94 °C for 7 min, and then 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s for 25 cycles, followed by 7-min extension at 72 °C using a PTC-100 programmable thermal controller (MJ Research Inc., Waltham, MA, USA). The expected 905 bp product for the wild-type allele, which spans the transposon insertion site, and 510 bp product for the mutant allele, which corresponds to a unique junctional product consisting of host and transposon sequences, were separated on a 1% agarose gel.

Probe design and synthesis

DNA templates (712 bp in length, 768–1478) were synthesized by standard PCR strategy. To enable synthesis of antisense and sense cRNA probes, T7 and SP6 RNA polymerase promoters were added at the extremities of the sense and antisense templates, respectively. The following primers were used:

Sense probes

Rn_TRPC4-F1-T7ext:

5′-CGCTATAATACGACTCACTATAGGGAGA**CCTAGATCAGACACGGAGTTCCAGAGAGCT**-3′

Rn_TRPC4-R1:

5′-CAGGCGGAGGGAACTGAAGATGTTT-3′

Anti-sense probes

TRPC4-F1:

5′-CCTAGATCAGACACGGAGTTCCAGAGAGCT-3′

TRPC4-R1-SP6ext:

5′-GCATTAATTTAGGTGACACTATAGAAGCG**CAGGCGGAGGGAACTGAAGATGTTT**-3′

The cRNA transcripts were validated by cold RNA probe synthesis and agarose gel electrophoresis prior to radiolabeling with ^{35}S-UTP (> 1000 Ci/mmol; Cat. #NEG039H, Perkin Elmer LAS Canada, Inc., London, Ontario, Canada).

Tissue preparation, ISH and autoradiography

Rat pups (postnatal day p5) were included in localization studies comparing Fisher WT (n = 3), *TRPC4*$^{+/-}$ (n = 6), and *TRPC4*$^{-/-}$ (n = 6) rats. Adult rats were anesthetized (isoflurane), and decapitated. Spinal cord (thoracic, lumbar and sacral segments) and left and right dorsal root ganglia (DRG) including lumbar L6 and