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Cannabinoid receptor antagonists AM251 and AM630 activate TRPA1 in sensory neurons

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ABSTRACT

Cannabinoid receptor antagonists have been utilized extensively *in vivo* as well as *in vitro*, but their selectivity has not been fully examined. We investigated activation of sensory neurons by two cannabinoid antagonists – AM251 and AM630. AM251 and AM630 activated trigeminal (TG) sensory neurons in a concentration-dependent fashion (threshold 1 μM). AM251 and AM630 responses are mediated by the TRPA1 channel in a majority (90–95%) of small-to-medium TG sensory neurons. AM630 (1–100 μM), but not AM251, was a significantly more potent agonist in cells co-expressing both TRPA1 and TRPV1 channels. We next evaluated AM630 and AM251 effects on TRPV1- and TRPA1-mediated responses in TG neurons. Capsaicin (CAP) effects were inhibited by pre-treatment with AM630, but not AM251. Mustard oil (MO) and WIN55,212-2 (WIN) TRPA1 mediated responses were also inhibited by pre-treatment with AM630, but not AM251 (25 uM each). Co-treatment of neurons with WIN and either AM630 or AM251 had opposite effects: AM630 sensitized WIN responses, whereas AM251 inhibited WIN responses. WIN-induced inhibition of CAP responses in sensory neurons was reversed by AM630 pre-treatment and AM251 co-treatment (25 μM each), as these conditions inhibit WIN responses. Hindpaw injections of AM630 and AM251 did not produce nocifensive behaviors. However, both compounds modulated CAP-induced thermal hyperalgesia in wild-type mice and rats, but not TRPA1 null-mutant mice. AMs also partially regulate WIN inhibition of CAP-induced thermal hyperalgesia in a TRPA1-dependent fashion. In summary, these findings demonstrate alternative targets for the cannabinoid antagonists, AM251 and AM630, in peripheral antihyperalgesia which involve certain TRP channels.

1. Introduction

Cannabinoids exert profound peripherally meditated thermal and mechanical antinociception and antihyperalgesia in several animal pain models (Calignano et al., 1998; Ibrahim et al., 2003; Johanek et al., 2001; Johanek and Simone, 2004; Khasabova et al., 2008; LaBuda et al., 2005; Malan et al., 2001; Richardson et al., 1998). There is an agreement that cannabinoids utilize multiple pathways to evoke peripheral antinociceptive and antihyperalgesic activities. These pathways are mediated via either metabotropic CB1 (Agarwal et al., 2007; Richardson et al., 1998), CB2 receptors (Malan et al., 2003), or ionotropic transient receptor potential (TRP) channels (Akopian et al., 2009, 2008; Patwardhan et al., 2006b; Sagar et al., 2004). Cannabinoids appear to exert this peripheral antinociception and antihyperalgesia by either directly inhibiting sensory neurons (Ahluwalia et al., 2000; Akopian et al., 2008; Patwardhan et al., 2006b) or modulating sensory neuron function indirectly via recruitment of non-neuronal peripheral cells such as keratinocytes (Ibrahim et al., 2005), mast cells (Jonsson et al., 2006; Samson et al., 2003), or macrophages (McCoy et al., 1999).

Peripheral mechanisms of cannabinoid actions have been evaluated using both pharmacological (Johanek and Simone, 2004; Malan et al., 2001; Richardson et al., 1998) and genetic approaches (Agarwal et al., 2007; Akopian et al., 2008; Ibrahim et al., 2006). It is important to note that the local injection of CB1 and CB2 receptor antagonists often occur at relatively high concentrations (high μM-low mM range) in several pain models (Fox et al., 2001; Ibrahim et al., 2005, 2006; Malan et al., 2001). In contrast, *in vitro* binding assay demonstrated that IC_{50} for AM251, a CB1 antagonist and AM630, a CB2 antagonist are ≈8 nM and ≈31 nM, respectively (Gatley et al., 1996; Hosohata et al., 1997). The specificity of these antagonists beyond CB1 and CB2 has not been evaluated in detail. This is an important question, as additional actions of these antagonists on ion channels involved in nociception could lead to a non-CB1/CB2 mechanism for

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antagonizing cannabinoid-mediated inhibition of peripheral nociceptors. This concern is supported by the observation that the cannabinoid agonist, WIN 55, 212-2, produced equivalent antinociception in wild-type (WT) and $CB1^{-/-}$ mice (Ibrahim et al., 2006). In contrast, the antinociceptive effects of WIN55, 212-2 were blocked by the CB_1 receptor-selective antagonist SR141716A (Bridges et al., 2001; Fox et al., 2001). One possibility for this discrepancy is that the SR141716A could be non-selective for CB1 at high doses. It is possible that TRP channels could also be modulated by cannabinoid antagonists. Thus, an antagonist of the putative anandamide transporter, AM404, gates TRPV1 (Zygmunt et al., 2000). In this study, we investigated activation of sensory neurons by a wide range of concentrations of the frequently used cannabinoid antagonists, AM251 (for CB1) and AM630 (for CB2).

2. Methods

2.1. Animals and primary sensory neuron culture

Breeding colonies for TRPA1 and TRPV1 channel null-mutant mice were provided by Dr. Kevin Kwan and The Jackson Laboratory, respectively (Caterina et al., 2000; Kwan et al., 2006). TRPA1 null-mutant mice were generated on the B6129P1/F2J background. Sprague–Dawley rats, 45–60 days old, were obtained from a commercial breeder (Charles River Laboratories, Inc., Wilmington, MA or Harlan, Indianapolis, IN, USA). All experiments conformed to protocols approved by the University Texas Health Science Center at San Antonio (UTHSCSA) Animal Care and Use Committee (IACUC). We followed guidelines issued by the National Institutes of Health and the Society for Neuroscience to minimize the number of animals used.

The animals were deeply anaesthetized with isoflurane and subsequently sacrificed. The trigeminal ganglia (TG) sensory neuron culture was generated as previously described (Akopian et al., 2007; Salas et al., 2009). Neurons were plated at low-density on poly-D-lysine/laminin coated coverslips or plates (Clontech, Palo Alto, CA). Cells were maintained in the presence or absence of 100 ng/ml NGF-7.02S (Harlan, Indianapolis, IN) as specified for each experiment. Ca^{2+}-imaging and patch clamp electrophysiology were performed 24–72 h after plating.

2.2. Constructs and heterologous expression in CHO cells

Expression plasmids of TRPV1 (accession number – NM031982) in pcDNA3 (Invitrogen, Carlsbad, CA) and TRPA1 (NM177781) in pcDNA5/FRT (Invitrogen) were used. Expression constructs with a visual marker (green fluorescent protein expressing pEGFP-N1 from Clontech) were delivered into Chinese hamster ovary (CHO) cells using PolyFect (Qiagen, Valencia, CA) according to manufacturers' protocols. CHO cells were subjected to experimental procedures within 24–48 h after transfection.

2.3. CGRP release assay

CGRP release assay of TG neurons was performed as previously described (Patwardhan et al., 2005, 2006a). Briefly, after two initial washes, a 15 min baseline sample was collected. Cultured TG neurons were then pre-treated or co-treated with drugs for 15 min and samples were collected after exposure to WIN. All the supernatants were collected for analysis of iCGRP content by radioimmunoassay (RIA). The basal release was typically 6–8 fmol per well. RIA was performed as previously (Garry et al., 1994; Patwardhan et al., 2006a). Primary antibody against CGRP (final dilution 1:1,000,000) was kindly donated by Dr. M.J. Iadarola (NIDCR/NIH).

2.4. Ca^{2+} imaging in TG neurons and CHO cells

The Ca^{2+} imaging experiments and ratiometric data conversion were performed as previously described (Akopian et al., 2007). The net changes in Ca^{+2} influx were calculated by subtracting the basal $[Ca^{+2}]_i$ (mean value collected for 60 s prior to addition of the first compound) from the peak $[Ca^{+2}]_i$ value achieved after exposure to the drugs. Ca^{2+} accumulations above 50 nM were considered positive. This minimal threshold criterion was established by application of 0.1% DMSO as a vehicle.

2.5. Electrophysiology

Recordings were made in whole-cell perforated patch voltage clamp (holding potential (V_h) of −60 mV) configuration at 22–24 °C from the somata of small-to-medium sized cultured TG neurons (15–40 pF) or CHO cells. Data were acquired and analyzed using an Axopatch 200B amplifier and pCLAMP9.0 software (Molecular Devices). Recording data were filtered at 0.5 kHz and sampled at 2 kHz. Access resistance (R_s) was compensated (40–80%) where appropriate up to the value of 13–18 MΩ. Data were rejected when R_s changed >20% during recording, leak currents were >50 pA, or input resistance was <200 MΩ. Currents were considered positive when their amplitudes were 5-fold bigger than displayed noise (in root mean square).

Standard external solution (SES) contained (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 D-glucose and 10 HEPES, pH 7.4. The pipette solution consisted of (in mM): 140 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 EGTA and 10 HEPES pH 7.3. The pipette solution for the perforated patch configurations consisted of (in mM): 140 KCl, 1 $MgCl_2$, 10 HEPES pH 7.3 and 250 μg/ml amphotericin B (Sigma, St. Louis, MO). Drugs were applied using a fast, pressure-driven and computer controlled 8-channel system (ValveLink8; AutoMate Scientific, San Francisco, CA).

2.6. Behavioral assays

Sprague–Dawley rats, 45–60-days old, and wild-type (WT) and TRPA1 null-mutant (TRPA1 KO) mice were used in behavioral assays. Two types of behavior assays were conducted. First, drug-induced nocifensive behavior was measured by observations of licking and flinching behavior over a 15 min time period. Licking and flinching was represented as spent time by the animals during the behavior (i.e. licking and flinching). Drug concentrations are specified in the "Legends to figures".

Second, capsaicin (CAP)-induced thermal hyperalgesia was utilized as a pain model (Patwardhan et al., 2006b). Thermal withdrawal latencies were measured using methods described previously (Hargreaves et al., 1988). The vehicle for cannabinoids and CAP was 5% DMSO and 5% Tween-80 (Johanek et al., 2001). After habituation and collection of basal withdrawal latencies, animals were injected ipl with the indicated drug combination at −15 min, then injected with CAP (10 μg for rats; 1 μg for mice) at time point "0", with measurement of paw withdrawal latencies at 5 and 10 min for evaluation of CAP induced thermal hyperalgesia. Thermal responses in mice were measured at 5 min points after CAP administration. All responses were collected by observers blinded to treatment allocation.

2.7. Data analysis

GraphPad Prism 4.0 (GraphPad, San Diego, CA) was used for statistical analysis. The data in Figs were given as mean ± standard error of the mean (SEM), with the value of n referring to the number of analyzed cells or trials for each group. All experiments were performed at least in triplicate. A significant difference between groups was assessed by one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison post-hoc test. In studies comparing two groups, data were analyzed using a paired or unpaired t-test. A difference was accepted as significant when $p < 0.05$, <0.01 or <0.001 and are identified by *, ** and ***, respectively.

3. Results

3.1. Cannabinoid antagonists AM251 and AM630 activate TG sensory neurons

The application of cannabinoid receptor antagonists AM251 and AM630 (10 μM each) activated a robust Ca^{2+} accumulation in a subset (≈35–40%) of TG neurons. The AM251 and AM630-evoked Ca^{2+} influxes into TG sensory neurons were concentration-dependent, and fitted using Hill's equation (Fig. 1A). The EC_{50} for AM251 and AM630 were 7.37 μM and 15.6 μM, respectively, although AM630 exhibited about four-fold increased efficacy compared with AM251. We next evaluated whether the presence of NGF (100 ng/ml) in culture media altered the magnitude of AM251 and AM630 responses. Fig. 1B demonstrates that the 72 h-exposure of TG neurons to NGF significantly increased AM251 and AM630 responses.

To independently replicate the findings by Ca^{2+}-imaging, AM251- and AM630-gated whole-cell currents (I_{AM251} and I_{AM630}) were measured. A wide range of concentrations (0.1–50 μM) of AM251 and AM630 are able to generate currents in TG sensory neurons (Fig. 1C). The activation threshold for I_{AM251} and I_{AM630} were 0.1 and 1 μM, respectively, although I_{AM630} was substantially larger than I_{AM251} at concentrations above 10 μM (Fig. 1C). Further, I_{AM630} had visibly faster activation and desensitization kinetics (Fig. 1D). Altogether, AM630 and AM251 are able to activate a subset of TG neurons with different efficacies.