Published in final edited form as:

Neurochem Int. 2010 August ; 57(1): 76–83. doi:10.1016/j.neuint.2010.05.002.

Activation of TRPC6 channels promotes endocannabinoid biosynthesis in neuronal CAD cells

Tamera K. Bardell and Eric L. Barker

Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University School of Pharmacy and Pharmaceutical Sciences, West Lafayette, IN 47907-2091

Abstract

Calcium influx activates biosynthesis of the endogenous cannabinoids 2-arachidonyl glycerol (2-AG) and anandamide (AEA). The calcium channel involved with endocannabinoid synthesis and release in neurons is still unknown. The canonical TRP (TRPC) channels are calcium-permeable channels that are a homology-based subdivision of the broader class of TRP channels. TRPC3, 6, and 7 are G-protein-gated nonselective cation channels that have been localized to lipid rafts and shown to colocalize with caveolin 1. Because endocannabinoid synthesis has been found to occur “on demand” in a calcium-dependent manner and has been linked to lipid rafts, we explored the potential role of transient receptor potential (TRP) channels in this process. Previously, we observed that after metabolism AEA and arachidonic acid (ArA) can be recycled into new endocannabinoid molecules. Consistent with these previous findings, we found that Cath.a differentiated (CAD) cells pretreated with radiolabeled ArA exhibited a robust increase in 2-AG release in response to TRPC stimulation with the diacylglycerol (DAG) analogue, 1-oleoyl-2-acetyl-*sn*-glycerol (OAG). Furthermore, cells pretreated with [³H]AEA produced a significant amount of AEA and 2-AG upon stimulation of TRPC channels. This process was not mediated through protein kinase C activation. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis revealed that only TRPC6 was present in the CAD cells. siRNA-induced knockdown of TRPC6 in the CAD cells abolished OAG-stimulated production of the endocannabinoids. This evidence suggests that TRPC6 may be capable of promoting endocannabinoid synthesis in neuronal cells.

Keywords

TRP channels; anandamide; cannabinoid

1. Introduction

There is considerable clinical and societal interest in drugs impacting the cannabinoid system. The major psychoactive ingredient in *Cannabis sativa*, Δ^9 -tetrahydrocannabinol (Δ^9 -THC), elicits effects on the central nervous system that could be used therapeutically for conditions including pain, appetite loss, asthma, fever, and autoimmune diseases (Gaoni and Mechoulam, 1971; Martin, 1986; Dewey, 1986). Cannabinoids such as Δ^9 -THC activate two cannabinoid

© 2010 Elsevier Ltd. All rights reserved.

Corresponding Author: Eric L. Barker, Ph.D., 575 Stadium Mall Drive, West Lafayette, IN, 47907-2091, Office: 765-494-9940, Fax: 765-494-1414, barkerel@purdue.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

receptors, CB1 and CB2, found in the nervous system and immune system, respectively (Matsuda et al., 1990; Munro et al., 1993). Endocannabinoids are the endogenous agonists of the CB1 and CB2 receptors. *N*-arachidonyl ethanolamine (anandamide, AEA) and 2-arachidonyl glycerol (2-AG) are the two most studied endocannabinoids and elicit responses similar to the cannabinoid Δ^9 -THC (Devane et al., 1992; Mechoulam et al., 1995).

Although many aspects of the endocannabinoid system have been well studied, many questions remain about the processes involved with AEA and 2-AG signaling. There are several models describing pathways involved with the synthesis of both of these endocannabinoids; however, less is known about actual molecular triggers for the synthesis (Yates and Barker, 2009). 2-AG and AEA are thought to be stored in the membrane as phospholipid precursors (Piomelli, 2003). 2-AG is synthesized from the precursor diacylglycerol (DAG) through the calcium-dependent action of sn1-specific DAG lipase (Bisogno et al., 2003), although there is evidence for phospholipase C-independent mechanisms for 2-AG formation as well (Bisogno et al., 1999; Carrier et al., 2004). There is strong support for AEA being synthesized from a precursor, *N*-arachidonyl phosphatidylethanolamine (NAPE) a calcium-dependent enzyme NAPE phospholipase D (NAPE PLD) (Di Marzo et al., 1994; Okamoto et al., 2004; Placzek et al., 2008). Like 2-AG, calcium-independent pathways for AEA biosynthesis have been described (Vellani et al., 2008). In most of these pathways, calcium entry is thought to be a critical stimulus for endocannabinoid synthesis due to the calcium-dependent nature of the process (Sugiura et al., 1996; Prescott and Majerus, 1983), but the calcium channels involved have yet to be identified.

Transient receptor potential (TRP) channels are calcium-permeable channels (Dalrymple et al., 2002). Canonical TRP (TRPC) channels are a homology-based subdivision of the broader class of TRP channels (Montell, 2001). TRPC3/6/7 are G-protein-gated nonselective cation channels that has been localized to lipid rafts and shown to co-localize with caveolin 1 (Isshiki and Anderson, 1999; Lockwich et al., 2000; Lockwich et al., 2001; Montell, 2001). Furthermore, activation of these channels has been shown to elevate intracellular calcium levels in a variety of cells (Soboloff et al., 2005; Spassova et al., 2004). These channels are found in a subfamily of canonical transient receptor potential channels that are also sensitive to direct activation by DAG (Montell, 2001). In the present studies, we explored the potential role of the TRPC3/6/7 family of channels in endocannabinoid biosynthesis in the neuronal Cath.a differentiated (CAD) cell line.

2. Materials and Methods

2.1. Cell Culture

CAD cells were maintained in a 50/50 mixture of HAMS-F12/Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 1% penicillin/streptomycin, 5% Fetal Clone I and 5% Bovine Calf Serum in a humidified CO₂ incubator at 37 °C. Cath.a (central adrenergic tyrosine hydroxylase-containing) cells are a CNS catecholaminergic cell line that has been isolated from a brain tumor in a transgenic mouse. These cells not only show physical characteristics that are similar to primary neuronal cells when they are differentiated, but they also have been shown to express neuronal markers such as neurofilaments, synaptophysin, and voltage-gated channels (Na⁺, K⁺, and Ca²⁺) (Suri et al., 1993). Cath.a differentiated (CAD) cells form short processes and upon further differentiation due to serum starvation the processes grow and cellular division stops. In addition to this, CAD cells have lost an immortalizing oncogene that is present in the parental cell line, and express neuron-specific proteins that were not present prior to differentiation (Qi et al., 1997). Due to these similarities to primary neuronal cells, CAD cells are a relevant model to use in neuropharmacological studies.

2.2. Sucrose Density Gradient

CAD cells were seeded into four 150 × 25 mm tissue culture plates 24 hours prior to experiment. Cells were assayed beginning with three washes of 20 ml ice cold phosphate buffered saline (PBS). Next, sucrose gradient lysis buffer (50 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl, and 0.5% Triton×100, pH 7.4) was added to each plate and incubated on ice for 20 min. The cells were scraped from the plates, similar lysates were combined, and they were added to 80% sucrose resulting in a 40% sucrose/lysate solution. The 40% solution was then transferred into an ultra-clear centrifuge tube (Beckman) followed by careful layering of 4 ml of 30% and 2 ml of 5% sucrose. The samples were then fractionated by ultracentrifugation at 200,000 × *g* for 18 h at 4°C. The samples were then taken as 1 ml fractions from the top. The fractions were subjected to SDS-PAGE and Western blot analysis.

2.3. Western blot analysis

Samples (whole cell lysate or sucrose density gradient fractions) were prepared for sodium dodecyl sulfate (SDS) -polyacrylamide gel electrophoresis (PAGE) by first determining the protein concentration by use of a BCA protein assay (Pierce). Laemmli buffer (5% bromophenol blue, 5% β-mercaptoethanol (BME), 62.5 mM Tris-HCl, 20% glycerol, and 2% SDS) was added to each sample in a 1:1 ratio. The samples were mixed at room temperature for 10 min and boiled for 5 min before being loaded into a 4–20% Tris-HCL gel for SDS-PAGE. The Mini-Protean 3 system (BioRad) was used for electrophoresis at 140 V for 45 min. The proteins were then transferred onto a polyvinylidene difluoride (PVDF) membrane at 60 V over 3 hours using the BioRad Mini Trans-Blot system. Membranes were placed in a blocking solution (PBS with 0.1% Tween-20 and 5% dry milk) at 4 °C overnight.

Expression of TRPC6, and β-actin were detected by use of α-TRPC6 goat polyclonal antibody (Santa Cruz), and β-actin monoclonal 1° antibody, respectively. These were followed by either horseradish peroxidase-labeled goat anti-rabbit antibody (BioRad), horseradish peroxidase-labeled rabbit anti-goat antibody (BioRad), or horseradish peroxidase-labeled goat anti-mouse antibody (BioRad) and enhanced chemiluminescence (ECL) detection reagents. Membranes were then exposed to X-ray film and developed.

2.4. Endocannabinoid Synthesis upon OAG stimulation

CAD cells were seeded into a 6 well plate at 1.5×10^5 cells/well. After 18–24 h culture media was replaced with media supplemented with either 5 nM [^3H] AEA or 5 nM [^3H]arachidonic acid (ArA) for 5 hours. Cells were then washed twice with KRH buffer supplemented with 2 mM calcium chloride and 0.1% fatty acid-free bovine serum albumin (KRH/Ca/BSA) and once with KRH supplemented with only 2 mM calcium chloride (KRH/Ca).

Endocannabinoid synthesis was stimulated by 100 μM OAG. Following a 10 min incubation at 37 °C, reaction buffer was removed and added to a scintillation vial with 10 ml Eco-Lite scintillation fluid to measure total tritium. The rest of the reaction buffer was transferred to a 15 ml polypropylene conical tube and 1.5 ml of 2:1 chloroform/methanol was added. The tubes were vortexed and centrifuged at 2000 × *g*, 4°C, 10 min. The organic layer was transferred to a glass tube and 1 ml of ice cold chloroform was added to the sample. The tubes were vortexed and centrifuged again, and the second organic layer was combined with the first. Unlabeled AEA, 2-AG, and ArA standards were added to the organic layer samples, and they were evaporated under an argon stream and then stored at –20°C overnight.

To determine the role of lipid rafts in endocannabinoid synthesis, CAD cells were washed as above, and then subjected to nystatin (25 μg/ml)/ progesterone (10 μg/ml) or methyl-β-cyclodextrin (5 mM) treatment for 30 min prior to OAG stimulation. To investigate the possible role of PKC in the endocannabinoid synthesis, phorbol 12-myristate 13-acetate (PMA) (PKC

agonist) and GF109203X (GFX) (specific PKC inhibitor) were used in the place of OAG. Cells were treated with PMA (1 μ M) for 15 min at 37 °C or 100 nM GFX at 37 °C for 5 min prior to OAG stimulation to stimulate and inhibit PKC activity as described previously (Kwan et al., 2006).

For thin layer chromatography (TLC) analysis of endocannabinoids, samples were dissolved in ice-cold chloroform and spotted on a silica TLC plate as previously described (Parrish and Nichols, 2006). This method has proven useful for simple detection of endocannabinoid biosynthesis (Placzek et al., 2008). The TLC plate was subjected to 11:5 isooctane/ethylacetate mobile phase for one hour followed by one hour in an iodine chamber to visualize the location of standards. The samples were removed from the plate, and the silica containing each sample was added to an individual scintillation vial with 10 ml Ecolite. The vials were mixed at room temperature for three days before the radioactivity was determined with an LS 6500 Multi-purpose Scintillation Counter (Beckman Coulter). Data were analyzed using GraphPad Prism v3.0.

2.5. 3' Rapid Amplification of cDNA Ends (RACE) Amplification

CAD cells were plated to confluence in 100 mm cell culture dishes or 6 well plates. The next day, total RNA was extracted using TRIzol reagent (Invitrogen). Briefly, cells were lysed with TRIzol followed by separation of the RNA with chloroform. The RNA found in the aqueous phase was removed and precipitated with isopropanol and then stored in 75% ethanol. In preparation for PCR, the RNA was dried and dissolved in diethylpyrocarbonate (DEPC) - treated water. Before cDNA synthesis, the RNA was incubated at 55–60°C for 10 min, and the concentration was determined by spectroscopy.

Conversion of this RNA into cDNA was performed using 3' RACE protocol (Invitrogen). In short, 5 μ g of total RNA was added to 10 μ M adapter primer (AP) and incubated at 70°C for 10 min. After chilling on ice for 1 min, the PCR buffer, $MgCl_2$, deoxyribonucleotide triphosphate (dNTP) mix, and dithiothreitol (DTT) were added to create the following solution: 20 mM Tris-HCl (pH 8.4 at 22°C), 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM DTT, 500 nM AP, 500 μ M each dATP, dCTP, dGTP, dTTP, and 1–5 μ g of RNA. This was mixed and equilibrated at 42°C for 2–5 min before SuperScript II reverse transcriptase was added. The reaction was incubated at 42°C for 50 min and terminated at 70°C for 15 minutes. RNase H was added to the mixture on ice and then incubated for 20 minutes at 37°C. For the polymerase chain reaction (PCR), the following components were added to the cDNA synthesis reaction product: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 200 nM of two gene specific primers (GSP) (sense and anti-sense), 200 μ M each dATP, dCTP, dGTP, dTTP, and 0.04 to 0.1 unit/ μ l *Taq* DNA polymerase. The GSP's were designed specifically for TRPC3 (Sense: 5'-ccctcagatcatatctgaagg-3'/Antisense: 5'-ctcacatctcagcacactggg-3'), TRPC6 (Sense: 5'-cctttactcctactacattggcgc-3'/Antisense: 5'-gcggcttctctccagcttggc-3'), or TRPC7 (Sense: 5'-ggcctttatgatcggaatgttcaacc-3'/Antisense: 5'-ccttgccttggttcacccgc-3') (Integrated DNA Technologies, INC). This mixture was incubated at 94°C for 3 min followed by 30 cycles of PCR (94°C for 45 sec, 49°C for 30 sec, 72°C for 3 min). The amplified sample was analyzed with agarose gel electrophoresis and ethidium bromide staining.

2.6. Stealth RNAi transfections

CAD cells were plated in 6-well culture plates. At 60% confluence, cells were transfected using the siLentFect lipid reagent protocol (BioRad). For optimization, 10 nM Stealth RNAi (TRPC6 MSS212126, MSS212127, MSS212128; Invitrogen) was used for 48 hours. Each of the oligos was used separately as well as in combination with the other two. Two negative controls were used: Stealth™ RNAi Negative Control LO GC (for MSS212127) and Stealth™ RNAi Negative Control Medium GC (for MSS212126 and MSS212128). These mock sequences

contained low and medium GC content respectively, and matched no known sequences in the vertebrate genome. Knockdown was achieved by transfection of just one of the three oligos for 48 hours and efficiency determined by Western blot analysis.

2.7. Overexpression of TRPC6 in CAD cells

CAD cells were plated in 6-well culture plates. At ~90% confluence, cells were transfected with vector (pcDNA3) or human TRPC6 cDNA (kindly provided by Dr. Craig Montell, Johns Hopkins University) using Lipofectamine 2000 (Invitrogen) per the manufacturer's protocol. Cells were assayed for endocannabinoid biosynthesis 48 hours after transfection.

2.8. Statistical Analysis

One sample t tests were performed on all of the biosynthesis and release experimental data to determine statistical significance. A hypothetical mean value of 1 was used as a reference for these t tests. Unpaired two-tailed t tests were also used to analyze the biosynthesis and release experiments when multiple treatments were used. A confidence interval of 95% was used to determine significance. All tests performed using GraphPad Prism v.3.0 (GraphPad Software, San Diego, CA).

3. Results

3.1. OAG stimulation causes endocannabinoid synthesis

The TRPC3/6/7 family of calcium channels can be activated by the diacylglycerol (DAG) analogue, 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) (Dietrich et al., 2005a). DAG analogues appear to directly activate TRPC3/6/7 channels independent of protein kinase C (PKC) (Hofmann et al., 1999). To assess the involvement of this subfamily of TRPC channels in endocannabinoid biosynthesis, CAD cells were stimulated with KRH buffer supplemented with 2 mM calcium chloride (KRH/Ca, control) or OAG (100 μ M). Endocannabinoid synthesis experiments were performed in cells metabolically pre-labeled with [3 H]AEA or [3 H]ArA. To label the CAD cells, the tritiated precursor was added to the medium where it could be incorporated into the cells. Previously, we observed that the tritiated precursors (AEA or ArA) could be taken up into the cognate mast cell line RBL-2H3, metabolized, and then reincorporated into membrane phospholipids to be recycled into new endocannabinoids (AEA or 2-AG) (Placzek et al., 2008). The question was whether a similar phenomenon could be observed for the neuronal CAD cells.

OAG stimulation induced a ~2-fold increase in lipids co-migrating with AEA and 2-AG standards on our TLC plates. These endocannabinoids were released into the medium when the CAD cells were prelabeled with [3 H]AEA (Fig. 1). Based upon the specific activity of the prelabel, we estimate that the 2-AG levels released from these cells is 50–100 times higher than AEA consistent with previous findings in brain where 2-AG is found to be approximately 150 times more abundant than AEA (Sugiura et al., 1995; Stella et al., 1997). Interestingly, when cells were pre-labeled with [3 H]ArA, a ~2-fold increase in 2-AG release was observed with no detectable release of AEA (Fig. 2). Interestingly, ionomycin, the non-selective calcium ionophore which is used routinely to stimulate endocannabinoid production, did not stimulate synthesis of either AEA or 2-AG in the CAD cells (data not shown) suggesting that the CAD cells might have reduced responsiveness for intracellular store calcium release. Analysis of lysates from unstimulated CAD cells revealed, in the case of AEA, that the [3 H]AEA used for prelabeling had been completely metabolized suggesting that intact [3 H]AEA was not being stored within the cells (data not shown). We do recognize that the TLC method does not provide definitive evidence for the identities of the lipids being released into the media, however, co-migration with our standards suggest that if these compounds are not AEA or 2-

AG, then they are close structural analogs consistent with other endocannabinoid-like molecules.

3.2. PKC stimulation or inhibition did not have an effect on endocannabinoid synthesis

PKC is involved in many pathways throughout the cell including activation/inhibition of the TRPC family of calcium channels. DAG, and therefore its analogue, OAG, are commonly used as activators of PKC (Venkatachalam et al., 2003; Venkatachalam et al., 2004; Soboloff et al., 2007). To investigate the involvement of PKC in the OAG-induced stimulation of endocannabinoid synthesis and release, we utilized a PKC agonist, phorbol 12-myristate 13-acetate (PMA), and a specific PKC inhibitor, GF109203X (GFX), in our synthesis and release experiments. To determine if the OAG was activating PKC instead of TRPC6, we stimulated the CAD cells for 15 minutes with 1 μ M PMA at 37°C. The PMA stimulation had no effect on endocannabinoid synthesis and release in CAD cells (Fig. 1 and Fig. 2). Also, inhibition of PKC with 100 nM GFX for 5 minutes prior to OAG stimulation did not have any inhibitory effects on the response seen upon OAG stimulation (Fig. 1 and Fig. 2). Overall, these results suggest that PKC is not involved in OAG-induced stimulation of endocannabinoid synthesis and release.

3.3. Disruption of lipid rafts reduces OAG-induced endocannabinoid synthesis

Depletion of cellular cholesterol is a common method for disrupting membrane microdomains such as lipid rafts and caveolae. Pretreatment of cells with nystatin/progesterone or methyl- β -cyclodextrin can be used to deplete cells of cholesterol by disruption of synthesis and transport (Rothberg et al., 1992; Subtil et al., 1999). Indeed, we have previously observed that these treatments deplete membrane cholesterol in CAD cells (McFarland et al., 2008). There is growing evidence linking endocannabinoid signaling and lipid rafts (Bari et al., 2005; Maccarrone et al., 2009; Rimmerman et al., 2008; Szoke et al., 2010) and specifically evidence supporting that endocannabinoid synthesis and release occurs at or near caveolae (Placzek et al., 2008; McFarland and Barker, 2005; McFarland et al., 2004; McFarland et al., 2008; Rimmerman et al., 2008). Therefore, the effects of the disruption of caveolae on OAG-induced endocannabinoid biosynthesis and release were assessed.

Nystatin/progesterone or methyl- β -cyclodextrin pre-treatment of CAD cells metabolically labeled with [3 H]AEA revealed a significant attenuation of AEA and 2-AG release (Fig. 3A, B). Likewise, CAD cells prelabeled with [3 H]ArA exhibited a significant attenuation in 2-AG release after nystatin/progesterone treatment (Fig. 3C, D). These data suggest an important role of cholesterol, and therefore possibly lipid raft membrane microdomains, in the TRPC-mediated synthesis and release of endocannabinoids.

3.4. 3' RACE and PCR determine the presence of TRPC6 in CAD cells

All three TRPC channels in the TRPC3/6/7 family can be stimulated by OAG (Dietrich et al., 2005a; Hofmann et al., 1999). We believe one of these three channels is involved in endocannabinoid biosynthesis and release. We performed polymerase chain reaction (PCR) using a 3' rapid amplification of cDNA ends (RACE) system to determine which of the three channels were present in CAD cells in order to help determine which could potentially be involved in endocannabinoid biosynthesis. To do so we designed specific primers (Integrated DNA Technologies, INC) for each of the three TRPC channels. The 3' RACE reaction revealed that only TRPC6 was expressed in CAD cells (Fig. 4).

3.5. Subcellular localization of TRPC6 in CAD cells

Recent evidence has been found that supports the theory that endocannabinoid biosynthesis, which requires calcium, occurs near or at lipid rafts or caveolae (McFarland and Barker,

2005; McFarland et al., 2004; Rimmerman et al., 2008). Some classical TRP channels, specifically TRPC1 and TRPC3, have not only been found in lipid rafts, but have also been found to co-localize with caveolin 1, a protein that localizes to caveolae (Isshiki and Anderson, 1999; Lockwich et al., 2000; Montell, 2001). To determine the expression and localization of TRPC6 in CAD cells, sucrose density fractionation followed by SDS-PAGE and Western blot analysis were performed. The TRPC6 channels expressed in CAD cells were not found to localize to lipid rafts/caveolae, but were found in the more dense fractions of CAD cell membranes (Fig. 5).

3.6. RNAi knockdown of TRPC6 attenuated OAG stimulated endocannabinoid synthesis

Since TRPC6 was the only TRPC3/6/7 channel family member observed in CAD cells, we further examined the functional involvement of TRPC6 in the endocannabinoid response to OAG stimulation. We used RNAi knockdown of TRPC6 to determine if we could abolish the OAG response. TRPC6 specific RNAi oligomers were transfected into CAD cells to knockdown the expression of TRPC6. We accomplished knockdown of the TRPC6 as determined by Western blot analysis (Fig. 6A). A negative control medium GC content siRNA oligonucleotide was used in parallel RNAi experiments to account for non-specific effects of the RNAi process.

After the knockdown of TRPC6 with siRNA oligomers (Invitrogen), endocannabinoid synthesis and release experiments were performed with wild-type and siRNA-treated cells to verify that OAG was stimulating endocannabinoid synthesis via TRPC6 channels. The cells treated with TRPC6-directed siRNA did not produce a similar endocannabinoid release to OAG stimulation as wild-type CAD cells (Fig. 6B,C). The [³H]AEA-derived endocannabinoid release in the siRNA-treated cells in response to OAG was absent compared to control and wild-type cells (Fig. 6B,C).

In addition to knockdown of a protein, over-expression studies can be used to infer functional consequences of receptor activation. Over-expression of human TRPC6 in CAD cells led to ~2-fold increase in both AEA and 2-AG production when cells were stimulated with OAG (Fig. 7).

4. Discussion

Many of the mechanisms that make up the endocannabinoid system have been investigated but are still not completely understood. Endocannabinoid biosynthesis and release is one of these mechanisms. The known characteristics of endocannabinoid biosynthesis and release include being calcium-dependent and an “on demand” process (Sugiura et al., 2002). Recent evidence has implicated intracellular storage organelles (lipid droplets) and fatty acid binding proteins in the intracellular transport of endocannabinoids (Kaczocha et al., 2009; Kaczocha et al., 2010). These cellular components could play a role both in the uptake and inactivation of endocannabinoids as well as their biosynthesis and release. There is also evidence that endocannabinoid biosynthesis and release occurs at or near caveolae in the plasma membrane (McFarland and Barker, 2005; McFarland et al., 2004; McFarland et al., 2008). One of the questions that remain is what calcium channel is responsible for the calcium influx necessary for endocannabinoid biosynthesis and release to occur.

TRPC channels are calcium permeable ion channels. There is evidence that three of the seven channels in the TRPC channel family (TRPC1, TRPC3, and TRPC4) have been found in caveolae and form complexes with caveolin 1 (Isshiki et al., 2002; Murata et al., 2007; Torihashii et al., 2002; Lockwich et al., 2000; Montell, 2001). Of these channels, TRPC3 can be activated by DAG, the precursor of one of the most common endocannabinoids, 2-AG (Bisogno et al., 1999; Bisogno et al., 1997; Di Marzo et al., 1999) and OAG, a DAG analogue

(Dietrich et al., 2005a; Dietrich et al., 2005b; Venkatachalam et al., 2003). However, TRPC4 has been shown to form a complex with another DAG-sensitive TRPC channel, TRPC6 (Yuan et al., 2007).

Our functional data in which OAG stimulates the release of endocannabinoids, AEA and 2-AG, suggests the involvement of one of the three TRPC channels in the DAG-sensitive family, TRPC3, TRPC6, or TRPC7 in endocannabinoid biosynthesis and release. The presence of the TRPC6 calcium channels in CAD cells was confirmed by our 3' RACE reactions and Western blots. However, there are other factors that could be causing the response to OAG that is observed in CAD cells. One pathway in particular involves PKC activation by OAG. OAG has been used to stimulate a PKC response, and PKC can affect TRPC channels both by activation and inhibition (Soboloff et al., 2007; Venkatachalam et al., 2003; Venkatachalam et al., 2004). To ensure that the OAG response in CAD cells was not due to PKC activation, we used both a PKC agonist (PMA) and inhibitor (GF109203X) in our biosynthesis and release experiments. Our data suggest that there is no involvement of PKC in this pathway of endocannabinoid biosynthesis and release, because stimulating PKC did not promote the response and inhibition of PKC did not decrease the OAG-mediated release of AEA or 2-AG. We did not observe an increase in endocannabinoid biosynthesis and release when GFX was used to inhibit PKC in the absence of OAG stimulation. This finding suggests that there is not an inhibitory effect of PKC on TRPC channel basal activity in CAD cells. Kwan *et. al.* have shown previously that not only does PKC inhibit TRPC3 by direct phosphorylation (Kwan et al., 2004), but also by indirect inhibition of TRPC3 through PKG (Kwan et al., 2006).

To further examine the hypothesis that endocannabinoid biosynthesis and release occurs at or near lipid rafts/caveolae (McFarland et al., 2004; McFarland and Barker, 2005; Rimmerman et al., 2008), we used a pre-treatment of nystatin/progesterone to disrupt lipid rafts in our biosynthesis and release experiments. We found that by depleting the CAD cells of cholesterol, and thereby disrupting lipid rafts, we could significantly attenuate the synthesis and release of both AEA and 2-AG. This finding is consistent with our previous results demonstrating that disruption of lipid raft domains also reduces endocannabinoid biosynthesis (Placzek et al., 2008). There was only a partial reduction of the response in both conditions which suggests that lipid rafts may not be required for this process but may have a role in increasing the efficiency of the biosynthetic process. Our lab has previously provided evidence of AEA metabolites recycling back to lipid rafts/caveolae (McFarland et al., 2004; McFarland et al., 2008), so it could be very possible that biosynthesis of AEA is happening within the lipid rafts. The membrane microdomains may allow for biosynthesis by gathering all of the components necessary for biosynthesis to occur. On the contrary, lipid rafts, specifically caveolae, could be involved in the release of endocannabinoids via an exocytotic method not yet determined.

To investigate a role for TRPC channels in endocannabinoid biosynthesis and release via lipid rafts, we fractionated the cells via sucrose density gradient to determine the localization of the TRPC channels in CAD cells. We did not observe TRPC6 channels in the lipid raft fraction that we isolated via sucrose density gradient, like we had expected based on previous work (Isshiki and Anderson, 1999; Lockwich et al., 2000; Montell, 2001). In CAD cells, the channels were found in the more dense fractions of the sucrose density gradient, which mainly include plasma membrane and endoplasmic reticulum proteins (Chatterjee et al., 1976; Bansal et al., 1985). Our data support previous findings by Lemonnier *et. al.* suggesting TRPC channels are present in the ER in a complex called SERCA (Lemonnier et al., 2006). Many other groups share the theory that TRPC channels form a complex with additional ER proteins including Homer 1, IP3R, and STIM1 (Kim et al., 2006; Yuan et al., 2007).

We have presented evidence that suggests that TRPC6 may be involved in the biosynthesis and release of endocannabinoids. The biosynthesis and release of endocannabinoids has been

studied in depth (Battista et al., 2004; Bisogno et al., 2005; Di Marzo et al., 1999; Sugiura et al., 2002). The biosynthesis of the two most common endocannabinoids, AEA and 2-AG, have been found to be calcium-dependent (Prescott and Majerus, 1983; Sugiura et al., 1996). We have shown the presence of TRPC6 calcium channels in CAD cells. Although TRPC6-mediated calcium permeation may be voltage-dependent, TRPC6 activation has been shown to elevate intracellular calcium concentrations to 50–100 nM (Estacion et al., 2006). DAG lipase, which is responsible for 2-AG biosynthesis, is activated by high concentrations of calcium (mM) suggesting that for TRPC6-mediated calcium influx to promote 2-AG synthesis as we observed, the local concentration of calcium must be rather high (Kondo et al., 1998). Future studies should focus on determining whether TRPC6 and DAG lipase are in close proximity to each other allowing for the requisite concentrations of calcium to be achieved for 2-AG synthesis. In particular, it now appears that DAG lipase- α plays a major role in retrograde signaling associated with 2-AG in the brain (Tanimura et al., 2010). In this light, a closer look at cellular localizations of TRPC6 and DAG lipase- α would seem warranted.

To determine if TRPC6 could be the calcium channel that is involved in endocannabinoid synthesis, we activated the channels and measured the biosynthesis and release of both AEA and 2-AG. We stimulated the DAG-sensitive TRPC channel family (TRPC3/6/7) (Dietrich et al., 2005a; Hofmann et al., 1999) by treatment with the DAG analogue, OAG (Dietrich et al., 2005a; Dietrich et al., 2005b; Venkatachalam et al., 2003). When we stimulated CAD cells with OAG, we found that endocannabinoid biosynthesis and release of both AEA and 2-AG were induced. However, the specific endocannabinoid that was released was dependent on the source of precursor arachidonate. When cells were metabolically labeled in the presence of [^3H]AEA, both AEA and 2-AG were produced. Only 2-AG was produced when cells were metabolically labeled with [^3H]ArA. These results suggest that the mechanism of endocannabinoid biosynthesis and release of endocannabinoids is not only tightly regulated (Schmid, 2000), but is specific to the precursor available. In a separate study, we examined more closely the metabolic fate of AEA and ArA discovering that AEA is metabolized into a diverse set of molecules that are distinct from the metabolites of ArA (Placzek et al., 2010). These lipidomic studies comparing AEA and ArA metabolism are consistent with our observation and suggest that ArA may not be efficiently incorporated in AEA precursors over the time course of the present experimental design. Indeed, subsequent analysis of our mass spectrometry data revealed that the AEA precursor, glycerophospho-N-arachidonoyl ethanolamine, is observed when cells are labeled with deuterated AEA, but not deuterated ArA (data not shown) (Simon and Cravatt, 2008). Molecular studies using RNAi to knockdown TRPC6 and over-expression of human TRPC6 provided support for the conclusion that TRPC6 activation is able to promote endocannabinoid biosynthesis. As TRPC channels play an important role in sensory neuron conduction and development, their contributions to the endocannabinoid system warrant further study as potential therapeutic targets (Birnbauer, 2009).

Abbreviations

TRP	transient receptor potential
TRPC	canonical TRP
2-AG	2-arachidonoyl glycerol
AEA	anandamide
TLC	thin layer chromatography
DAG	diacylglycerol
OAG	1-oleoyl-2-acetyl- <i>sn</i> -glycerol

RT-PCR reverse transcriptase-polymerase chain reaction

Acknowledgments

This work was supported by the National Institutes of Health grant R21 DA018112 (E.L.B.).

References

- Bansal VS, Hattori H, Orihel D, Kanfer JN. Distribution of selected phospholipid modifying enzymes in rat brain microsomal subfractions prepared by density gradient zonal rotor centrifugation. *Neurochem. Res* 1985;10:439–451. [PubMed: 2987722]
- Bari M, Battista N, Fezza F, Finazzi-Agrò A, Maccarrone M. Lipid rafts control signaling of type-1 cannabinoid receptors in neuronal cells. Implications for anandamide-induced apoptosis. *J Biol Chem* 2005;280:12212–12220. [PubMed: 15657045]
- Battista N, Fezza F, Maccarrone M. Endocannabinoids and their involvement in the neurovascular system. *Curr. Neurovasc. Res* 2004;1:129–140. [PubMed: 16185189]
- Birnbaumer L. The TRPC class of ion channels: a critical review of their roles in slow, sustained increases in intracellular Ca(2+) concentrations. *Ann. Rev. Pharmacol. Toxicol* 2009;49:395–426. [PubMed: 19281310]
- Bisogno T, Howell F, Williams G, Minassi A, Cascio MG, Ligresti A, Matias I, Schiano-Moriello A, Paul P, Williams EJ, Gangadharan U, Hobbs C, Di Marzo V, Doherty P. Cloning of the first sn1-DAG lipases points to the spatial and temporal regulation of endocannabinoid signaling in the brain. *J. Cell Biol* 2003;163:463–468. [PubMed: 14610053]
- Bisogno T, Ligresti A, Di Marzo V. The endocannabinoid signalling system: biochemical aspects. *Pharmacol. Biochem. Behav* 2005;81:224–238. [PubMed: 15935454]
- Bisogno T, Melck D, DePetrocellis L, Di Marzo V. Phosphatidic acid as the biosynthetic precursor of the endocannabinoid 2-arachidonoylglycerol in intact mouse neuroblastoma cells stimulated with ionomycin. *J. Neurochem* 1999;72:2113–2119. [PubMed: 10217292]
- Bisogno T, Sepe N, Melck D, Maurelli S, DePetrocellis L, Di Marzo V. Biosynthesis, release and degradation of the novel endogenous cannabimimetic metabolite 2-arachidonoylglycerol in mouse neuroblastoma cells. *Biochem. J* 1997;322:671–677. [PubMed: 9065792]
- Carrier EJ, Kearn CS, Barkmeier AJ, Breese NM, Yang W, Nithipatikom K, Pfister SL, Campbell WB, Hillard CJ. Cultured rat microglial cells synthesize the endocannabinoid 2-arachidonoylglycerol, which increases proliferation via a CB2 receptor-dependent mechanism. *Mol. Pharmacol* 2004;65:999–1007. [PubMed: 15044630]
- Chatterjee SK, Kim U, Bielat K. Plasma membrane associated enzymes of mammary tumours as the biochemical indicators of metastasizing capacity. Analyses of enriched plasma membrane preparations. *Br. J. Cancer* 1976;33:15–26. [PubMed: 175819]
- Dalrymple A, Slater DM, Beech D, Poston L, Tribe RM. Molecular identification and localization of Trp homologues, putative calcium channels, in pregnant human uterus. *Mol. Hum. Reprod* 2002;8:946–951. [PubMed: 12356946]
- Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D, Mandelbaum A, Etinger A, Mechoulam R. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science* 1992;258:1946–1949. [PubMed: 1470919]
- Dewey WL. Cannabinoid pharmacology. *Pharmacol. Rev* 1986;38:151–178. [PubMed: 3529128]
- Di Marzo V, Bisogno T, De Petrocellis L, Melck D, Orlando P, Wagner JA, Kunos G. Biosynthesis and inactivation of the endocannabinoid 2-arachidonoylglycerol in circulating and tumoral macrophages. *Eur. J. Biochem* 1999;264:258–267. [PubMed: 10447696]
- Di Marzo V, Fontana A, Cadas H, Schinelli S, Cimino G, Schwartz JC, Piomelli D. Formation and inactivation of endogenous cannabinoid anandamide in central neurons. *Nature* 1994;372:686–691. [PubMed: 7990962]

- Dietrich A, Kalwa H, Rost BR, Gudermann T. The diacylglycerol-sensitive TRPC3/6/7 subfamily of cation channels: functional characterization and physiological relevance. *Pflugers Arch* 2005a; 451:72–80. [PubMed: 15971081]
- Dietrich A, Schnitzler M, Kalwa H, Storch U, Gudermann T. Functional characterization and physiological relevance of the TRPC3/6/7 subfamily of cation channels. *Naunyn Schmiedeberg Arch. Pharmacol* 2005b;371:257–265. [PubMed: 15909153]
- Estacion M, Sinkins WG, Jones SW, Applegate MA, Schilling WP. Human TRPC6 expressed in HEK 293 cells forms non-selective cation channels with limited Ca^{2+} permeability. *J Physiol* 2006;572:359–377. [PubMed: 16439426]
- Gaoni Y, Mechoulam R. The isolation and structure of delta-1-tetrahydrocannabinol and other neutral cannabinoids from hashish. *J. Am. Chem. Soc* 1971;93:217–224. [PubMed: 5538858]
- Hofmann T, Obukhov AG, Schaefer M, Harteneck C, Gudermann T, Schultz G. Direct activation of human TRPC6 and TRPC3 channels by diacylglycerol. *Nature* 1999;397:259–263. [PubMed: 9930701]
- Isshiki M, Anderson RG. Calcium signal transduction from caveolae. *Cell Calcium* 1999;26:201–208. [PubMed: 10643558]
- Isshiki M, Ying Y, Fujita T, Anderson RGW. A molecular sensor detects signal transduction from caveolae in living cells. *J. Biol. Chem* 2002;277:43389–43398. [PubMed: 12177060]
- Kaczocha M, Glaser ST, Deutsch DG. Identification of intracellular carriers for the endocannabinoid anandamide. *Proc Natl Acad Sci U S A* 2009;106:6375–6380. [PubMed: 19307565]
- Kaczocha M, Glaser ST, Chae J, Brown DA, Deutsch DG. Lipid droplets are novel sites of N-acylethanolamine inactivation by fatty acid amide hydrolase-2. *J Biol Chem* 2010;285:2796–2806. [PubMed: 19926788]
- Kim JY, Zeng W, Kiselyov K, Yuan JP, Dehoff MH, Mikoshiba K, Worley PF, Muallem S. Homer 1 mediates store- and inositol 1,4,5-trisphosphate receptor-dependent translocation and retrieval of TRPC3 to the plasma membrane. *J. Biol. Chem* 2006;281:32540–32549. [PubMed: 16887806]
- Kondo S, Kondo H, Nakane S, Kodaka T, Tokumura A, Waku K, Sugiura T. 2-Arachidonoylglycerol, an endogenous cannabinoid receptor agonist: identification as one of the major species of monoacylglycerols in various rat tissues, and evidence for its generation through CA^{2+} -dependent and -independent mechanisms. *FEBS Lett* 1998;429:152–156. [PubMed: 9650580]
- Kwan HY, Huang Y, Yao X. Regulation of canonical transient receptor potential isoform 3 (TRPC3) channel by protein kinase G. *Proc. Natl. Acad. Sci. USA* 2004;101:2625–2630. [PubMed: 14983059]
- Kwan HY, Huang Y, Yao X. Protein kinase C can inhibit TRPC3 channels indirectly via stimulating protein kinase G. *J. Cell Physiol* 2006;207:315–321. [PubMed: 16331690]
- Lemonnier L, Trebak M, Lievreumont JP, Bird GS, Putney JW Jr. Protection of TRPC7 cation channels from calcium inhibition by closely associated SERCA pumps. *FASEB J* 2006;20:503–505. [PubMed: 16401641]
- Lockwich T, Singh BB, Liu X, Ambudkar IS. Stabilization of cortical actin induces internalization of transient receptor potential 3 (Trp3)-associated caveolar Ca^{2+} signaling complex and loss of Ca^{2+} influx without disruption of Trp3-inositol trisphosphate receptor association. *J. Biol. Chem* 2001;276:42401–42408. [PubMed: 11524429]
- Lockwich TP, Liu X, Singh BB, Jadowiec J, Weiland S, Ambudkar IS. Assembly of Trp1 in a signaling complex associated with caveolin-scaffolding lipid raft domains. *J. Biol. Chem* 2000;275:11934–11942. [PubMed: 10766822]
- Maccarrone M, DeChiara V, Gasperi V, Viscomi MT, Rossi S, Oddi S, Molinari M, Musella A, Finazzi-Agrò A, Centonze D. Lipid rafts regulate 2-arachidonoylglycerol metabolism and physiological activity in the striatum. *J Neurochem* 2009;109:371–381. [PubMed: 19187444]
- Martin BR. Cellular effects of cannabinoids. *Pharmacol. Rev* 1986;38:45–74. [PubMed: 2872689]
- Matsuda LA, Lolait SJ, Brownstein MJ, Young AC, Bonner TI. Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature* 1990;346:561–564. [PubMed: 2165569]
- McFarland MJ, Bardell TK, Yates ML, Placzek EA, Barker EL. RNA interference-mediated knockdown of dynamin 2 reduces endocannabinoid uptake into neuronal dCAD cells. *Mol. Pharmacol* 2008;74:101–108. [PubMed: 18436710]

- McFarland MJ, Porter AC, Rakhshan F, Rawat DS, Gibbs RA, Barker EL. A role for caveolae/lipid rafts in the uptake and recycling of the endogenous cannabinoid anandamide. *J. Biol. Chem* 2004;279:41991–41997. [PubMed: 15292270]
- McFarland MJ, Barker EL. Lipid rafts: A nexus for endocannabinoid signaling? *Life Sciences* 2005;77:1640–1650. [PubMed: 15978630]
- Mechoulam R, Ben-Shabat S, Hanus L, Ligumsky M, Kaminski NE, Schatz AR, Gopher A, Almog S, Martin BR, Compton DR, et al. Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem. Pharmacol* 1995;50:83–90. [PubMed: 7605349]
- Montell C. Physiology, phylogeny, and functions of the TRP superfamily of cation channels. *Sci STKE* 2001 2001:RE1.
- Munro S, Thomas KL, Abu-Shaar M. Molecular characterization of a peripheral receptor for cannabinoids. *Nature* 1993;365:61–65. [PubMed: 7689702]
- Murata T, Lin MI, Stan RV, Bauer PM, Yu J, Sessa WC. Genetic evidence supporting caveolae microdomain regulation of calcium entry in endothelial cells. *J. Biol. Chem* 2007;282:16631–16643. [PubMed: 17416589]
- Okamoto Y, Morishita J, Tsuboi K, Tonai T, Ueda N. Molecular characterization of a phospholipase D generating anandamide and its congeners. *J. Biol. Chem* 2004;279:5298–5305. [PubMed: 14634025]
- Parrish JC, Nichols DE. Serotonin 5-HT_{2A} receptor activation induces 2-arachidonoylglycerol release through a phospholipase c-dependent mechanism. *Journal of Neurochemistry* 2006;99:1164–1175. [PubMed: 17010161]
- Piomelli D. The molecular logic of endocannabinoid signalling. *Nat. Rev. Neurosci* 2003;4:873–884. [PubMed: 14595399]
- Placzek EA, Cooper BR, Placzek AT, Chester JA, Davisson VJ, Barker EL. Lipidomic metabolism analysis of the endogenous cannabinoid anandamide (N-arachidonylethanolamide). *J.Pharm. Biomed. Anal.* 2010 in press, doi:10.1016/j.jpba.2010.03.035.
- Placzek EA, Okamoto Y, Ueda N, Barker EL. Mechanisms for recycling and biosynthesis of endogenous cannabinoids anandamide and 2-arachidonoylglycerol. *J. Neurochem* 2008;107:987–1000. [PubMed: 18778304]
- Prescott SM, Majerus PW. Characterization of 1,2-diacylglycerol hydrolysis in human platelets. Demonstration of an arachidonoyl-monoacylglycerol intermediate. *J. Biol. Chem* 1983;258:764–769. [PubMed: 6822511]
- Qi Y, Wang JK, McMillian M, Chikaraishi DM. Characterization of a CNS cell Line, CAD, in which morphological differentiation is initiated by serum deprivation. *Journal of Neuroscience* 1997;17:1217–1225. [PubMed: 9006967]
- Rimmerman N, Hughes HV, Bradshaw HB, Pazos MX, Mackie K, Prieto AL, Walker JM. Compartmentalization of endocannabinoids into lipid rafts in a dorsal root ganglion cell line. *Br. J. Pharmacol* 2008;153:380–389. [PubMed: 17965731]
- Rothberg KG, Heuser JE, Donzell WC, Ying YS, Glenney JR, Anderson RG. Caveolin, a protein component of caveolae membrane coats. *Cell* 1992;68:673–682. [PubMed: 1739974]
- Schmid HH. Pathways and mechanisms of N-acylethanolamine biosynthesis: can anandamide be generated selectively? *Chem. Phys. Lipids* 2000;108:71–87. [PubMed: 11106783]
- Simon GM, Cravatt BF. Anandamide biosynthesis catalyzed by the phosphodiesterase GDE1 and detection of glycerophospho-N-acyl ethanolamine precursors in mouse brain. *J. Biol. Chem* 2008;283:9341–9349. [PubMed: 18227059]
- Soboloff J, Spassova M, Hewavitharana T, He LP, Luncsford P, Xu W, Venkatachalam K, van Rossum DB, Patterson RL, Gill DL. TRPC channels: integrators of multiple cellular signals. *Handb. Exp. Pharmacol* 2007;575–591. [PubMed: 17217080]
- Soboloff J, Spassova M, Xu W, He LP, Cuesta N, Gill DL. Role of endogenous TRPC6 channels in Ca²⁺ + signal generation in A7r5 smooth muscle cells. *J. Biol. Chem* 2005;280:39786–39794. [PubMed: 16204251]
- Spassova MA, Soboloff J, He LP, Hewavitharana T, Xu W, Venkatachalam K, van Rossum DB, Patterson RL, Gill DL. Calcium entry mediated by SOCs and TRP channels: variations and enigma. *Biochim. Biophys. Acta* 2004;1742:9–20. [PubMed: 15590052]

- Stella N, Schweitzer P, Piomelli D. A second endogenous cannabinoid that modulates long-term potentiation. *Nature* 1997;388:773–778. [PubMed: 9285589]
- Subtil A, Gaidarov I, Kobylarz K, Lampson MA, Keen JH, McGraw TE. Acute cholesterol depletion inhibits clathrin-coated pit budding. *Proc. Natl. Acad. Sci. USA* 1999;96:6775–6780. [PubMed: 10359788]
- Sugiura T, Kobayashi Y, Oka S, Waku K. Biosynthesis and degradation of anandamide and 2-arachidonoylglycerol and their possible physiological significance. *Prostaglandins Leukot. Essent. Fatty Acids* 2002;66:173–192. [PubMed: 12052034]
- Sugiura T, Kondo S, Sukagawa A, Nakane S, Shinoda A, Itoh K, Yamashita A, Waku K. 2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain. *Biochem. Biophys. Res. Commun* 1995;215:89–97. [PubMed: 7575630]
- Sugiura T, Kondo S, Sukagawa A, Tonegawa T, Nakane S, Yamashita A, Ishima Y, Waku K. Transacylase-mediated and phosphodiesterase-mediated synthesis of N-arachidonylethanolamine, an endogenous cannabinoid-receptor ligand, in rat brain microsomes. Comparison with synthesis from free arachidonic acid and ethanolamine. *Eur. J. Biochem* 1996;240:53–62. [PubMed: 8797835]
- Suri C, Fung BP, Tischler AS, Chikaraishi DM. Catecholaminergic cell lines from the brain and adrenal glands of tyrosine hydroxylase-SV40 T antigen transgenic mice. *Journal of Neuroscience* 1993;13:1280–1291. [PubMed: 7680068]
- Sozke E, Börzsei R, Tóth DM, Lengi O, Helyes Z, Sándor Z, Szolcsányi J. Effect of lipid raft disruption on TRPV1 receptor activation of trigeminal sensory neurons and transfected cell line. *Eur J Pharmacol* 2010;628:67–74. [PubMed: 19958765]
- Tanimura A, Yamazaki M, Hashimoto Y, Uchigashima M, Kawata S, Abe M, Kita Y, Hashimoto K, Shimizu T, Watanabe M, Sakimura K, Kano M. The endocannabinoid 2-arachidonoylglycerol produced by diacylglycerol lipase α mediates retrograde suppression of synaptic transmission. *Neuron* 2010;65:320–327. [PubMed: 20159446]
- Torihashi S, Fujimoto T, Trost C, Nakayama S. Calcium oscillation linked to pacemaking of interstitial cells of Cajal: requirement of calcium influx and localization of TRP4 in caveolae. *J. Biol. Chem* 2002;277:19191–19197. [PubMed: 11897792]
- Vellani V, Petrosino S, DePetrocellis L, Valenti M, Prandini M, Magherini PC, McNaughton PA, Di Marzo V. Functional lipidomics. Calcium-independent activation of endocannabinoid/endovanilloid lipid signalling in sensory neurons by protein kinases C and A and thrombin. *Neuropharmacology* 2008;55:1274–1279. [PubMed: 18329052]
- Venkatachalam K, Zheng F, Gill DL. Regulation of canonical transient receptor potential (TRPC) channel function by diacylglycerol and protein kinase C. *J. Biol. Chem* 2003;278:29031–29040. [PubMed: 12721302]
- Venkatachalam K, Zheng F, Gill DL. Control of TRPC and store-operated channels by protein kinase C. *Novartis Found. Symp* 2004;258:172–185. [PubMed: 15104182]
- Yates ML, Barker EL. Inactivation and biotransformation of the endogenous cannabinoids anandamide and 2-arachidonoylglycerol. *Mol. Pharmacol* 2009;76:11–17. [PubMed: 19389920]
- Yuan JP, Zeng W, Huang GN, Worley PF, Muallem S. STIM1 heteromultimerizes TRPC channels to determine their function as store-operated channels. *Nat. Cell Biol* 2007;9:636–645. [PubMed: 17486119]

Figure 1. OAG treatment induced AEA and 2-AG biosynthesis and release when CAD cells were prelabeled with [³H]AEA
Prelabeling of CAD cells with [³H]AEA and treatments with OAG (100 μ M), PMA (1 μ M), or GF109203X (GFX; 100 nM) were performed as described in Materials and Methods. (A), AEA release and (B), 2-AG release measured using the TLC method described in Materials and Methods. Values shown are mean $\pm$ S.E.M. (n=3–8). *p<0.05, one sample t-test comparing to 1.0.

Figure 2. OAG treatment induced 2-AG biosynthesis and release when CAD cells were prelabeled with [^3H]ArA

Prelabeling of CAD cells with [^3H]ArA and treatments with OAG (100 μM), PMA (1 μM), or GF109203X (GFX; 100 nM) were performed as described in Materials and Methods. (A), AEA release and (B), 2-AG release measured using the TLC method described in Materials and Methods. Values shown are mean $\pm$ S.E.M. (n=3-8). *p<0.05, one sample t-test comparing to 1.0.

Figure 3. Nystatin/progesterone and methyl- β -cyclodextrin treatment attenuated endocannabinoid biosynthesis and release stimulated by OAG

Endocannabinoid synthesis experiments were performed by prelabeling CAD cells with $[^3\text{H}]\text{AEA}$ (A,B) or $[^3\text{H}]\text{ArA}$ (C,D) as described in Materials and Methods. Prior to stimulating the cells with OAG (100 μM), cells were subjected to a 30 minute treatment with either nystatin (25 $\mu\text{g}/\text{ml}$)/progesterone (10 $\mu\text{g}/\text{ml}$) (N/P) or methyl- β -cyclodextrin (5 mM)(M β CD). (A,C) AEA release and (B,D) 2-AG release measured using the TLC method described in Materials and Methods. Values shown are mean $\pm$ S.E.M. (n=4). *p<0.05, unpaired t-test.

Figure 4. TRPC6 was detected in CAD cells using 3'RACE

RNA was isolated from CAD cells using TRIzol reagent. The 3' RACE system cDNA amplification kit from Invitrogen was used to synthesize cDNA from the isolated RNA and amplify the TRPC3/6/7 genes as described in Materials and Methods. The amplified samples were analyzed by 1% agarose gel electrophoresis and ethidium bromide staining. Specific primers were designed to amplify fragments of TRPC3 (924 bp), TRPC6 (807 bp), or TRPC7 (789 bp). As a control, an actin primer set was used which generated a predicted fragment of 514 bp.

Figure 5. TRPC6 was present in the more dense fractions of CAD cells

CAD cells were fractionated via sucrose density gradient as described in Materials and Methods. Briefly, samples were analyzed by SDS-PAGE and Western blotting. Fraction #5 was not analyzed due to lack of protein content. Typically, a milky band appears at the interface of fractions 2 and 3 representing the detergent-insoluble caveolin-rich membrane fractions. The more dense fractions (6–10) contain cellular and membrane proteins such as fatty acid amide hydrolase, the endoplasmic reticulum marker BiP/GRP78, and the transferrin receptor (McFarland et al., 2004). An anti-TRPC6 goat polyclonal antibody (1:200) and a rabbit anti-goat (1:1000) horseradish peroxidase-labeled secondary antibody were used to detect TRPC6. The blot was stripped and re-blotted for actin. The anti-actin mAb (1:2000) and a goat anti-mouse (1:2000) horseradish peroxidase-labeled secondary antibody were used. Flotillin was also detected on previous blots of sucrose density gradients determining that lipid rafts are found at the interface of fractions 2 and 3 (data not shown). Labeling was followed by exposure with ECL detection reagents. (n=3)

Figure 6. RNAi-mediated knockdown of TRPC6 abolished OAG-mediated synthesis of AEA and 2-AG

(A), siRNA oligomers specific to TRPC6 was added to CAD cells as described in Materials and Methods, and knockdown allowed to proceed for 48 hours. TRPC6 expression was determined by Western blot analysis. (B,C), Functional analysis of TRPC6 knockdown. CAD cells were transfected with siRNA as described above and metabolically labeled with [³H] AEA. After a 10 minute treatment with 100 μM OAG, AEA (B) and 2-AG (C) biosynthesis and release were measured using the TLC method as described in Materials and Methods. Values shown are mean ± S.E.M. from 3 separate experiments performed in duplicate. *p<0.05, one sample t-test comparing to 1.0.

Figure 7. Over-expression of human TRPC6 enhanced OAG-mediated synthesis of AEA and 2-AG CAD cells were transfected as described in Materials and Methods. 48 hours after transfection, cells were metabolically labeled with $[^3\text{H}]\text{AEA}$ as described. After a 10 minute treatment with 100 μM OAG, endocannabinoid biosynthesis and release was measured using the TLC method as described in Materials and Methods. Values shown are mean $\pm$ S.E.M. from 3 separate experiments performed in duplicate. * $p < 0.05$, unpaired t-test.