이학 박사학위 논문

Studies on transient receptor potential vanilloid subtype 1 (TRPV1)-mediated degeneration of mesencephalic cells *in vivo* and *in vitro*

아주대학교 대학원

신경과학기술과정

김 상 룡

Studies on transient receptor potential vanilloid subtype 1 (TRPV1)-mediated degeneration of mesencephalic cells *in vivo* and *in vitro*

지도교수 진 병 관

이 논문을 이학 박사학위 논문으로 제출함.

2006년 2월

아주대학교 대학원

신경과학기술과정

김 상 룡

Studies on transient receptor potential vanilloid subtype 1 (TRPV1)-mediated degeneration of mesencephalic cells *in vivo* and *in vitro*

by

Sang Ryong Kim

A Dissertation Submitted to Graduate School of Ajou University in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

Supervised by

Byung Kwan Jin, Ph.D.

Neuroscience Graduate Program

The Graduate School, Ajou Univeristy

February, 2006

김상룡의 이학 박사학위 논문을 인준함.

심사위원장 백 은 주 인

심사위원 오 우 택 인

심사위원 조 은 혜 인

심사위원 이 용 범 인

심사위원 진 병 관 인

아주대학교 대 학 원

2005년 12월 16일

ACKNOWLEGEMENTS

Many thanks to my family including Dr. Jung for inspiration and support until accomplishment of Ph.D. I also want to express my thanks to my scientific adviser, Dr. Jin and members of thesis committee for their guidance, and to my friends including lab colleagues and members of brain disease research center in Ajou University.

- ABSTRACT -

Studies on Transient Receptor Potential Vanilloid Subtype 1 (TRPV1)-mediated Degeneration of Mesencephalic Cells *In Vivo* and *In Vitro*

Transient receptor potential vanilloid subtype 1 (TRPV1, also known as VR1) has an oligomeric structure formed by subunits having six transmembrane segments with a pore domain formed by the fifth and sixth transmembrane regions. This receptor is a nonselective cation channel activated by the vanilloids or products of lipoxygenases, and protein kinase C and phospholipase C mediate the sensitization of TRPV1. Moreover, the widespread distribution of TRPV1 including substantia nigra (SN) in the brain has suggested that this receptor plays a significant role in the central nervous system (CNS). However, little is known about toxicity via TRPV1 in the SN.

Intranigral injection of the TRPV1 agonist capsaicin (CAP) into the rat brain, or treatment of rat mesencephalic cultures with CAP, resulted in cell death of dopaminergic (DA) neurons, as visualized by immunocytochemistry. This in vivo and in vitro effect was ameliorated by the TRPV1 antagonist capsazepine (CZP) or iodo-resiniferatoxin (I-RTX), suggesting the direct involvement of TRPV1 in neurotoxicity. In cultures, both CAP and anandamide (AEA), an endogenous ligand for both TRPV1 and cannabinoid type 1 (CB1) receptors,

induced degeneration of DA neurons, increases in intracellular Ca^{2+} ($[Ca^{2+}]_i$), and mitochondrial damage, which were inhibited by CZP, the CB1 antagonist AM251 or the intracellular Ca^{2+} chelator BAPTA/AM. We also found that CAP or AEA increased mitochondrial cytochrome c release as well as immunoreactivity to cleaved caspase-3, and that the caspase-3 inhibitor z-DEVD-fmk protected DA neurons from CAP- or AEA-induced neurotoxicity. Additional studies demonstrated that treatment of mesencephalic cultures with CB1 receptor agonists HU210 or WIN 55,212-2 also produced degeneration of DA neurons and increases in $[Ca^{2+}]_i$, which were inhibited by CZP, AM251 or BAPTA/AM. The CAP-, AEA-, HU210-, or WIN 55,212-2-induced increases in $[Ca^{2+}]_i$ were dependent on extracellular Ca^{2+}, with significantly different patterns of Ca^{2+} influx. Surprisingly, CZP and AM251 reversed HU210-, WIN 55,212-2 or CAP-induced neurotoxicity by inhibiting Ca^{2+} influx, respectively, suggesting the existence of functional cross-talk between TRPV1 and CB1 receptors. Moreover, 12-hydroperoxyeicosatetraenoic acid (12-HPETE, known as TRPV1 agonist) produced via activation of CB1 receptors by HU210 or WIN 55,212-2 induced neuronal toxicity via activation of TRPV1 in mesencephalic cultures, and intranigral injection of 12-HPETE into the rat brain also resulted in neuronal cell death.

In addition, this study examined whether microglia express TRPV1 and activation of TRPV1 contributes to cell death of microglia. In cultures, RT-PCR,

Western blot analysis and immunocytochemical staining showed that microglia, but not astrocytes, expressing TRPV1 underwent cell death following the treatment with TRPV1 agonist CAP or RTX. Moreover, treatment with CAP or RTX induced cell death of immortalized human microglial cell line HMO6 expressing this receptor. CAP- or RTX-induced cell death of microglia was accompanied by increases in cytosolic Ca^{2+} concentration in the presence of extracellular Ca^{2+} and mitochondrial damage as well as mesencephalic neurons. This toxicity was also ameliorated by TRPV1 antagonists or BAPTA/AM, suggesting involvement of increases in cytosolic Ca^{2+} via influx through the direct activation of TRPV1. Additional study demonstrated that intranigral injection of CAP or 12-HPETE into the rat brain produced cell death of microglia, but not astrocytes in the SN, visualized by immunocytochemistry, and this in vivo effect was ameliorated by CZP or I-RTX, suggesting involvement of TRPV1 in the toxicity. This study is the first to demonstrate that the activation of TRPV1 and/or CB1 receptors mediates cell death of DA neurons, microglia express TRPV1, and activation of TRPV1 also mediates cell death of microglia. The findings in this study suggest that these two types of receptors, TRPV1 and CB1 receptors, may contribute to neurodegeneration in response to endogenous ligands such as AEA or 12-HPETE.

Key words : TRPV1, CB1 receptor, Intracellular Ca^{2+}, Dopaminergic neuron, Microglia, Anandamide, Capsaicin, Capsazepine, 12-HPETE, HU210

TABLE OF CONTENTS

PAGE OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATION

12-HPETE, 12-hydroperoxyeicosatetraenoic acid

AEA, anandamide

AP, anteroposterior

BCL, baicalein

BV, blood vessel

$[Ca^{2+}]_i$, intracellular Ca^{2+}

Calcein, calcein-acetoxymethyl ester

CAP, capsaicin

CB1, cannabinoid type 1

CNS, central nervous system

CZP, capsazepine

DA, dopaminergic

DMSO, dimethyl sulfoxide

DTT, dithiothreitol

DV, dorsoventral

Eth-1, ethidium homodimer-1

EtOH, ethanol

GABA, gamma amino butyric acid

GAD, glutamic acid decarboxylase

GAPDH, glyceraldehyde-3-phosphate dehydrogenase

GFAP, glial fibrillary acidic protein

HBSS, hanks' balanced salt solution

I-RTX, iodo-resiniferatoxin

LDH, lactate dehydrogenase

ML, mediolateral

NeuN, neuron-specific nuclear protein

PCR, polymerase chain reaction

PD, Parkinson's disease

PBS, phosphate-buffered saline

PMSF, phenylmethylsulfonyl fluoride

RTX, resiniferatoxin

SD, Sprague-Dawley

SN, substantia nigra

SNpc, substantia nigra pars compacta

SNr, Substantia nigra reticulata

TH, tyrosine hydroxylase

TRPV1, transient receptor potential vanilloid subtype 1

TUNEL, TdT-mediated dUTP Nick-End Labeling

VTA, ventral tegmental area

I. INTRODUCTION

1. Transient receptor potential vanilloid subtype 1 (TRPV1)

1.1. Character of TRPV1

Capsaicin (CAP), the hot principle contained in the plans of the genus Capsicum, is a powerful stimulus for a specific subset of primary sensory neurons in experimental animals and in humans. There is a large body of evidence indicating that the excitatory effect of CAP on sensory neurons is due to its ability to increase the open state of a channel previously defined as the 'CAP receptor'. The recent cloning of this molecular entity has revealed that it consists (Caterina et al., 1997) of a 426 amino-acid protein, which has been firstly termed vanilloid receptor-1 (VR1). The VR1 was soon recognised to belong to the TRP family of ion channels. TRPs have been subdivided into three main subclasses: TRPC, TRPM and TRPV. The CAP-activated TRPV1 belongs to the latter group (Montell et al., 2002). TRPV1 has an oligomeric structure formed by subunits having six transmembrane segments with a pore domain formed by the fifth and sixth transmembrane regions and intracellular N- and C-terminus (Ferrer-Montiel et al., 2004; Garcia-Sanz et al., 2004). This receptor is a nonselective cation channel activated by the vanilloids

(Caterina et al., 2001; Neubert et al., 2003; Cortright and Szallasi, 2004; Ferrer-Montiel et al., 2004; van der Stelt and Di Marzo, 2004; Kim et al., 2005), such as CAP, by its endogenous ligands, such as anandamide (AEA) or N-arachidonoyl-dopamine (Di Marzo et al., 2001a; Huang et al., 2002), and products of lipoxygenases, including 12-hydroperoxyeicosatetraenoic acid (12-HPETE; Hwang et al., 2000). TRPV1 is also gated by a variety of stimuli including extracellular acidic pH and noxious heat (Tominaga and Julius, 2000; Ferrer-Montiel et al., 2004).

1.1.1. Anandamide (AEA)

Anandamide (N-arachidonoylethanolamine, AEA, and known as TRPV1 agonist) is the main endocannabinoid described to date (Sugiura et al., 2002). They bind to both type 1 (CB1) and type 2 (CB2) cannabinoid receptors, thus mimicking some of the central and peripheral effects of D9-tetrahydrocannabinol, the psychoactive principle of hashish and marijuana (Mechoulam et al., 2002). Recently, AEA has been shown to also activate vanilloid receptors (De Petrocellis et al., 2001; Jordt and Julius, 2002). The effect of AEA via CB1 and CB2 receptors depends on its extracellular concentration, which is controlled by cellular uptake by a specific AEA membrane transporter (AMT), and intracellular degradation by the AEA-hydrolyzing enzyme fatty acid amide hydrolase

(FAAH). AMT (Hillard and Jarrahian, 2000) and FAAH (Bisogno et al., 2002) have been characterized in several mammalian cells and tissues. Moreover, the checkpoint in AEA synthesis is thought to be the N-acyl-phosphatidylethanolamines (NAPE)-hydrolyzing phospholipase D (PLD) (Hansen et al., 2000). Together with AEA and congeners, these proteins form the endocannabinoid system. AEA plays a number of roles in the central nervous system and in peripheral tissues (Di Marzo et al., 2002). In particular, AEA inhibits human cancer cell proliferation, being more generally involved in the control of cell survival and death with reported pro-apoptotic and antiapoptotic effects (Guzman et al., 2002). It is still under debate whether in the central nervous system AEA plays a role as neuroprotective or neurotoxic compound (Van der Stelt et al., 2002a and b). Intriguingly, TRPV1 stimulation with CAP, via the subsequent Ca^{2+} influx, leads to biosynthesis of AEA, which can activate both TRPV1 and CB1 receptors (Di Marzo et al., 1994; 2001b).

1.1.2. 12-hydroperoxyeicosatetraenoic acid (12-HPETE)

12-Lipoxygenase (12-LO) catalyzes the conversion of arachidonic acid (AA) to 12-hydroperoxyeicosatetraenoic acid (12-HPETE, also known as TRPV1 agonist), a 12-hydroperoxy fatty acid, which is then reduced to 12-hydroxyeicosatetraenoic acid (12-HETE) by glutathione peroxidase (Lehmann, 1994). There are three types of mammalian 12-LOs: platelet

(Nugteren, 1982), leukocyte (Reddy et al., 1994), and epidermal (Funk et al., 2002). Leukocyte 12-LO catalyzes the conversion of AA to 12-HPETE and, to a lesser extent, 15-HPETE (3:1 ratio) (Chen et al., 1994); or to 9-(S) hydroperoxy-9Z,11E-octadecadienoic acid and 13-hydroxyoctadecadienoic acid with linoleic acid as the substrate (Claeys et al., 1985), whereas platelet 12-LO only catalyzes the conversion of AA to 12-HPETE. The role of leukocyte 12-LO in promoting atherosclerosis recently has gained additional validation based on the elegant cross-breeding studies of 12-LO null mice that showed that targeted deletion of 12-LO reduces atherosclerosis (Funk et al., 2002). The 12-LO products, particularly the HETEs, have potent biologic effects and are integral to a number of signaling systems. 12-LO products activate transcription factor activating protein 1 (Rao et al., 1996) and nuclear factor kappa B (Stolz et al., 1996). 12-HETE also activates extracellular signal-regulated kinase (ERK) and NH_2-terminal c-Jun kinase (JNK) (Wen et al., 1997). 12-HETE in rat adrenal glomerulosa cells also can activate specific isoforms of protein kinase C (Natarajan et al. 1994). Moreover, 12-HPETE was implicated in induction of long-term depression at hippocampus (Feinmark et al., 2003), induced neurodegeneration in mesencephalic cultures (Canals et al., 2003), and recent data (Hwang et al., 2000; Shin et al., 2002) indicate that 12-HPETE or other LO products in rat dorsal root ganglia (DRGs) activae TRPV1 directly.

1.1.3. TRPV1 antagonists

TRPV1-expressing neurons have been proposed to play a major role in a large variety of diseases, including migraine and cluster headache (Goadsby, 2004), asthma and chronic obstructive pulmonary disease (Lee et al., 2005) and many others. Evidence for the involvement of TRPV1-expressing neurons in human disease originates from studies performed in animal models. There are also novel data obtained in humans by means of morphological localisation and semiquantitative assay of TRPV1 and desensitisation of sensory nerve terminals by repeated CAP application (Jones et al., 2004). While the use of TRPV1 antagonists is currently limited to animal models, results with CAP desensitisation in humans must be regarded with caution for two reasons. The first is due to the pungent action associated with CAP treatment, and an action that makes it difficult to control clinical trials. The second derives from the fact that CAP desensitization results from the specific action of the drug on TRPV1, but produces its effects (including the beneficial effect) by the defunctionalisation of the entire nerve terminals, which no longer responds, not only to TRPV1 agonists (protons, lipid derivatives, CAP, etc.) but also to all the other stimuli that act on different channels/receptors expressed on the nerve terminal. Thus, in this latter case, TRPV1 is of critical importance for the production of the CAP effect, but cannot offer any firm conclusion on the role of TRPV1 with

regard to the pathological condition under investigation. A detailed description of TRPV1 antagonists currently used in experimental animals and that may undergo scrutiny in clinical trials is reported in Table 1.

1.2. Distribution of TRPV1

Recent findings indicate that TRPV1 is expressed in at least three cellular compartments; in the cytoplasmic membrane, in the endoplasmic reticulum and in the cytoplasmic vesicles (Guo et al., 1999; Morenilla-Palao et al., 2004). While TRPV1s in the cytoplasmic membrane are responsible for the TRPV1-mediated effects, such as inward currents or transmitter release, those in the cytoplasmic vesicles seem to serve as a reserve, which can be quickly translocated to the cytoplasmic membrane, for example following PKC activation (Morenilla-Palao et al., 2004). The role of TRPV1 expressed by the endoplasmic reticulum is not clear. The finding that activation of these receptors by capsaicin or resiniferatoxin evokes Ca^{2+} mobilisation from intracellular stores shows that these receptors are also functional and they might be involved in the regulation of Ca^{2+} homeostasis (Karai et al., 2004; Marshall et al., 2003).

TRPV1 mRNA is highly expressed in a subset of primary sensory neurons with A-d and C fibres that respond to chemical, mechanical and thermal stimuli and, therefore, they are classified as polymodal nociceptos. TRPV1 mRNA is also expressed in diverse areas of the

Table 1. IC50 (nM) values of diverse TRPV1 antagonists

14 (Jerman et al., 2000) (ammoniated ruthenium oxychloride)	**Ruthenium Red**
912 (Rigoni et al., 2003)[a] (N-[2-(4-chlorophenyl)ethyl]-1,3,4,5- tetrahydro-7,8-dihydroxy-2H-2-benzazepine-2-carbothioamide)	**Capsazepine**
0.071 (Rigoni et al., 2003)[a] (6,7-deepoxy-6,7-didehydro-5-deoxy-21-dephenyl-21-(phenylmethyl)-daphnetoxin,20-(4-hydroxy-5-iodo-3-methoxybenzeneacetate)	**Iodo-resiniferatoxin**
638.6 (Appendino et al., 2003) (6-iodo-nordihy 8-methyl-N-vanillyl-trans-6-nonenamide)	**6-iodo-nordihydrocapsaicin**
7.5 (Gunthorpe et al., 2003) (N-(3-methoxyphenyl)-4-chlorocinnamide)	**SB 366791**
100 (Himmel et al., 2002) (l-enantiomers of the arginine-rich hexapeptide)	**l-R4W2**
7.8 (Lee et al., 2003)[b]	**N-[2-(3,4-dimethylbenzyl)-3-pivaloyloxypropyl]-N'-[3-fluoro-4-(methylsulphonylamino)benzyl] thiourea**
6–35 (Valenzano et al., 2003) (N-(4-tertiarybutylphenyl)-4-(3-cholorphyridin-2-yl)tetrahydropyrazine -1(2H)-carbox-amide)	**BCTC**

* IC50 values are referred to the inhibition of capsaicin-induced calcium uptake in: endogenous TRPV1 in rat DRG neurons. [a]Human TRPV1 HEK293 cell line. [b]rVR1 CHO cells.

central nervous system, including the limbic system (e.g. hippocampus, central amygdala and both medial and lateral habenula), striatum, hypothalamus, centromedian and paraventricular thalamic nuclei, substantia nigra (SN), reticular formation, locus coeruleus, cerebellum and inferior olive (Mezey et al., 2000; Roberts et al., 2004). There is also evidence that mRNA and protein of TRPV1 are produced and expressed in non-neuronal cells, including the epithelial cells of the urothelium (Birder et al., 2001), keratinocytes (Inoue et al., 2002) and epithelial cells of the palatal rugae (Kido et al., 2003).

1.3. Activation of TRPV1

The ionic event, triggered by TRPV1 gating, results in an excitatory effect on terminals of primary sensory neurons with the subsequent depolarisation of the nerve fibre and the initiation of action potential propagation. Orthodromic conduction of action potentials triggers reflex responses, including cough, voiding of the urinary bladder and in the gut contribute to peristalsis. Ca^{2+} influx into the nerve endings, driven either by antidromic conduction of action potential or directly by TRPV1 gating, causes the local release of neuropeptides, including calcitonin gene-related peptide (CGRP), substance P (SP) and neurokinin A (NKA). Activation of CGRP receptors and tachykinin (NK1, NK2 and NK3) receptors on effector cells, particularly at the vascular levels, causes a

series of inflammatory responses, collectively referred to as neurogenic inflammation (Geppetti and Holzer, 1996; Shin et al., 2002; Trevisani et al., 2002). In addition, activation of TRPV1 induces the accumulation of intracellular Ca^{2+} ($[Ca^{2+}]_i$) (Di Marzo et al., 2001a; Shin et al., 2003). Excessive mitochondrial Ca^{2+} load resulting from the treatment of sensory neurons with TRPV1 agonists has been shown to cause mitochondrial disruption, resulting in cell death (Olah et al., 2001; Shin et al., 2003; Kim et al., 2005).

1.3.1. Skin

The expression of TRPV1 in keratinocytes suggests that TRPV1 might be involved in the development of skin disorders. Capsaicin, through TRPV1 activates cyclooxygenase-2, in a Ca^{2+}-dependent manner and induces the release of interleukin-8 and prostaglandin E2 (Southall et al., 2003). It has been speculated that prostaglandin E2 released from keratinocytes may contribute to the activation of primary sensory neuronal terminals in the dermis (Southall et al., 2003).

1.3.2. Inner ear

Role of TRPV1 in the development of diseases, such as hyperacusis, tinnitus, vestibular hypersensitivity and some forms of episodic vertigo, has been suggested recently (Balaban et al., 2003). The proposal is based

on the findings that spiral and vestibular ganglionic cells, in addition to TRPV1 also express 5-lipoxygenase, the product of which has been suggested to be endogenous TRPV1 ligand and that increased lipoxygenase activity produces tinnitus. As capsaicin application to the scala tympany indeed induces elevation of the threshold of cochlear compound action potential generation, TRPV1 might be involved in the development of hyperacusis (Zheng et al., 2003).

1.3.3. Urinary tract

The significant role of TRPV1 in bladder disfunction has been well documented. Intravesical application of capsaicin or resiniferatoxin induces reflex activation of the bladder smooth muscle and neurogenic inflammation in the bladder wall (Maggi et al., 1989). Two mechanisms underlying the vanilloid-induced increased contractions have been postulated. According to the first hypothesis, capsaicin or resiniferatoxin directly activates capsaicin-sensitive primary sensory neurons in the subepithelial layer of the bladder, which in turn release substance P. Substance P then sensitises smooth muscle cells resulting in increased contractions (Quartara and Maggi, 1998). The second hypothesis is based on the recent finding that TRPV1 is also expressed by epithelial cells of the transitional epithelium, and activation of these TRPV1-expressing cells results in ATP release, which then activates P_2X_3 receptors

expressed by bladder afferents (Birder et al., 2001). Both mechanisms have been implicated in the development of micturition reflex. Stretching of the bladder wall during bladder filling activates TRPV1-expressing bladder afferents either directly or through the release of ATP from urothelial cells. In both cases, TRPV1 has been thought to act as a mechanotransducer. Caterina (2003) suggested that co-assembly of TRPV1 with mechano-responsive TRP proteins might underlay the mechanosensitivity of the capsaicin receptor in the bladder.

1.3.4. Airways

The sensitivity of the respiratory tract to capsaicin and other vanilloids is also well documented. Capsaicin-responsive afferents are either superficial fibres terminating in the mucosa or deep pulmonary fibres located in the alveolar septa (Paintal, 1973). The later types of fibres are associated with pulmonary blood vessels. Superficial fibres monitor the chemical environment of the airway mucosa and their activation results in cough, increased mucosal secretion and bronchoconstriction (Coleridge and Coleridge, 1984). The development of these effects involves substance P released from capsaicin-sensitive fibres (Maggi et al., 1991). Inflammation or altered responsiveness of immunocompetent cells located in the mucosa sensitises the mucosal nociceptors, which can significantly amplify the broncho- and secretomotor response leading to

the exacerbation of the pathological processes (Undem and Carr, 2001). This mechanism is considered as an important factor in the pathogenesis of asthma.

1.3.5. Brain

While vanilloids have been shown to modify activity in different areas of the central nervous system (Mezey et al., 2000; Al Hayani et al., 2001; Hajos and Freund, 2002; Roberts et al., 2004), very little is known about the physiological and pathophysiological role of TRPV1 in brain. However, significant role of TRPV1 in the central nervous system (CNS) have been reported in several regions of the rat brain, including the hypothalamus (Sasamura et al., 1998), locus ceruleus (Marinelli et al., 2002), and hippocampus (Huang et al., 2002). Activation of TRPV1 by treatment with CAP was found to induce increased glutamate release from nigral slices (Marinelli et al., 2003), to produce hypokinesia in parallel to decrease in the activity of nigrostriatal neurons (Di Marzo et al., 2001; de Lago et al., 2004), and to disrupt blood-brain barrier following ischemia-reperfusion (Hu et al., 2005). In addition, anandamide has been found to induce reduction in ambulation, stereotypies and exploration (De Lago et al., 2004). The anandamide-evoked effect can be significantly reduced by capsazepine. Moreover, capsazepine reverses the anandamide-evoked reduction of the 3,4-dihydroxyphenylacetic acid content of the caudate–putamen,

suggesting that TRPV1 activity decreases dopamine turnover in the basal ganglia, and anandamide decreases the stimulated dopamine release from nigrostriatal terminals.

2. Cannabinoid type 1 (CB1) receptor

2.1. Character and role of CB1 receptor

The CB1 receptor was first cloned as an orphan receptor from a rat cDNA library based on its homology to the bovine substanceKreceptor (Matsuda et al., 1990). CB1 belong to the superfamily of G protein–coupled receptors, coupling to inhibitory G proteins (Gi

ERROR: rangecheck
OFFENDING COMMAND: string

STACK:

66038
33018
32512
33019