ACTIVATION AND REGULATION OF TRP CHANNELS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Rui Xiao, M.S.

*****

The Ohio State University
2008

Dissertation Committee:
Professor Michael Xi Zhu, Adviser
Professor Anthony Brown
Professor John Enyeart
Professor Chen Gu

Approved by

_________________________
Adviser
Biophysics Graduate Program
ABSTRACT

Mammalian TRP superfamily is composed of 28 members. Based on the sequence homology and functional similarity, they are grouped into 6 subfamilies: canonical TRP (TRPC), vanilloid TRP (TRPV), melastatin TRP (TRPM), ankyrin TRP (TRPA), polycystin TRP (TRPP), and mucolipin TRP (TRPML). TRP channels display rather diverse tissue expression pattern and physiological functions. Many, if not all, TRP channels are regulated by multiple intracellular and extracellular factors, thus giving rise to the polymodal nature of TRP channels.

We mainly focused on three TRP channels: TRPV3 from TRPV subfamily; TRPC4 and TRPC5 from TRPC subfamily. For TRPV3, we performed three functional studies: first, we found that arachidonic acid (AA), together with other poly-unsaturated fatty acids (PUFAs), greatly potentiated TRPV3 channel function independent of its downstream metabolites or signaling pathways. Second, besides being potentiated by AA and other PUFAs, TRPV3 also displays an intrinsic unique functional property – sensitization upon prolonged or repetitive chemical or physical stimulations. We studied the molecular
mechanism underlying this unique feature and found that calcium plays a critical role from both intracellular and extracellular sides. Specifically, we identified an intracellular CaM-binding site at the N-terminus of TRPV3 (aa108-aa130). Disruption of CaM binding to this site greatly released TRPV3 from the inhibition by intracellular Ca\textsuperscript{2+}. Ca\textsuperscript{2+} also directly binds to the putative pore region of TRPV3 and blocks TRPV3 channel function. We identified a high-affinity binding site for extracellular Ca\textsuperscript{2+} in the pore-loop of TRPV3 – Asp641. Neutralization of this Asp to Asn largely removed the high-affinity Ca\textsuperscript{2+} block from the extracellular side. Third, a spontaneously occurring point mutation linked to rodent hair-less phenotype was mapped to Gly573 of TRPV3. We carried out detailed functional studies with G573C and G573S mutants of TRPV3, and discovered that these two point mutations cause constitutive channel activation and severe host cell death, which provides an important clue for the hairless phenotype.

Moreover, we studied the activation mechanism of TRPC4 and TRPC5. Strikingly, we found that in addition to the previously identified G\textsubscript{q/11} signaling pathway, another commonly considered inhibitory G protein-coupled receptor – G\textsubscript{i/o} signaling pathway was excitatory for the activation of TRPC4 and TRPC5. In fact, compared to G\textsubscript{q/11}, the G\textsubscript{i/o} pathway was even stronger for the activator of TRPC4/C5. We also demonstrated the synergistic effect between these two pathways. The full activation of TRPC4/C5 appeared to require both signaling pathways, indicating that TRPC4 and TRPC5 work as coincident detectors for the two important G protein signaling pathways. We further confirmed the
requirement of phospholipase C (PLC) and intracellular Ca\textsuperscript{2+} for the excitatory
effect of G\textsubscript{i/o}. More detailed studies will help us understand the precise underlying
mechanism of G\textsubscript{i/o}-induced TRPC4/C5 activation.

G\textsubscript{i/o} proteins are mostly abundant in brain, but their physiological roles in brain
remain largely unknown. Given that TRPC4/C5 are highly expressed in many
regions of mammalian brains, they may function as a target of G\textsubscript{i/o} signaling.
Since TRPC4 and TRPC5 are activated by G\textsubscript{i/o} and G\textsubscript{q/11} pathways in a
synergistic manner, TRPC4/C5 may work as coincident detectors of both
pathways.
Dedicated to my family and parents
ACKNOWLEDGMENTS

I am deeply indebted to my Ph.D. mentor, Dr. Michael X. Zhu, for his constant support, guidance, and encouragement throughout of my graduate study. He is really THE person who opened the door of science for me. Intellectually, I benefited a lot from his mentoring, and his two most important scientific rules were engraved in my mind: always set the positive and negative controls for your experiments; always try to get the highest signal-to-noise ratio for your experiments.

I am also grateful to Drs. Anthony Brown, John Enyeart, and Chen Gu for serving at my graduate committee. Their precious time and energy for advising my graduate research are highly appreciated.

I would like to express my appreciation to the committee members of Biophysics Graduate Program at OSU and especially Dr. Thomas Clanton. They recruited me into the wonderful graduate program and offered me the precious opportunity to receive an excellent scientific training.

I would like to thank all the labmates I have been working with, including but not restricted to Dina Zhu, Chunbo Wang, Jisen Tang, Hongzhen Hu, Jibin Tian, Craig Colton, Emilia Iscru, and Xuemei Hao. They provided an excellent environment and great help for my graduate education. Particulary, I want to
thank Hongzhen Hu, Chunbo Wang, Jinbin Tian, and Jisen Tang. Some of results presented in this thesis were provided by them.

Finally, I would like to express my sincere appreciation and love to my wife Haiyan Liu and my parents. They have always been my strongest back-up and support throughout the whole time.
VITA

December 6, 1977…………………………………….Born-Lianyuan, Hunan, China

1996-2000…………………………………………….B.S. in Biology, Beijing Normal University

2000-2003…………………………………….........M.S. in Cell Biology, Beijing Normal University

2003-present………………………………………..Ph.D. Candidate, Biophysics Graduate program, the Ohio State University

2008…………………………………………………..Outstanding Student Research Achievement Award, Biophysics Graduate Committee, the Ohio State University

2007…………………………………………………..National Institute of Neurological Disorders and Stroke (NINDS) Scholarship Winner, The Transient Receptor Potential Ion Channel Superfamily Meeting, Keystone Symposium
PUBLICATIONS

Research Publication


**FIELDS OF STUDY**

Major Field: Biophysics
# TABLE OF CONTENTS

| ABSTRACT                                      | ii |
| DEDICATIONS                                   | v  |
| ACKNOWLEDGMENTS                               | vi |
| VITA                                          | vii|
| LIST OF TABLES                                | xv |
| LIST OF FIGURES                               | xvi|
| ABBREVIATIONS                                | xix|

## CHAPTERS:

### Chapter 1 Introduction

1.1 A trip to TRP: historical overview ............. 1
1.2 General architecture of TRP channels .......... 4
1.3 Diverse functions of TRP channels ............... 6
  1.3.1 Physiological functions of TRPC channels ... 6
  1.3.2 Physiological functions of TRPV channels ... 9
  1.3.3 Physiological functions of TRPM channels ... 12
  1.3.4 Physiological functions of TRPA1 ............ 15
  1.3.5 Physiological functions of TRPP channels ... 16
  1.3.6 Physiological functions of TRPN channels ... 18
  1.3.7 Physiological functions of TRPML channels ... 18
  1.3.8 A generalized function for TRP channel superfamily ... 19
1.4 Activation and regulation mechanisms of TRP channels ... 19
  1.4.1 Activation and regulation of TRPC channels ... 20
    1.4.1.1 Information learnt from *Drosophila* TRPs ... 20
    1.4.1.2 Proteins that interact with TRPC channels ... 21
    1.4.1.3 The activation and regulation of TRPC1 ... 22
    1.4.1.4 The activation and regulation of TRPC2 ... 23
    1.4.1.5 The activation and regulation of TRPC3, C6, C7 ... 24
    1.4.1.6 The activation and regulation of TRPC4, C5 ... 27
    1.4.1.6.1 Receptor-mediated activation ... 27
    1.4.1.6.2 Store-operated activation ... 28
    1.4.1.6.3 Agonist-evoked activation ... 29
    1.4.1.6.4 Regulatory factors of TRPC4/5 .. 31
3.1. Introduction ........................................................................................................ 69
3.2. AA potentiates TRPV3 mediated [Ca^{2+}]i elevation ........................................... 71
3.3. AA potentiates 2-APB induced TRPV3 currents ................................................... 72
3.4. Functional properties of AA-potentiated TRPV3 current ........................................ 73
3.5. Single channel recordings of AA-potentiated TRPV3 currents in HEK293 cells ...................... 74
3.6. AA potentiates 2APB-induced TRPV3 current in *Xenopus* oocytes .............. 75
3.7. Dose-dependence of AA-potentiated TRPV3 current ............................................ 76
3.8. TRPV3 is directly potentiated by AA but not its downstream molecules ... 77
3.9. Other unsaturated fatty acids also potentiates TRPV3 ........................................ 78
3.10. Summary ........................................................................................................ 79

Chapter 4 Calcium plays a central role in the sensitization of TRPV3 .......... 90

4.1. Introduction ........................................................................................................ 90
4.2. Intracellular Ca^{2+} buffering strength affects the sensitization kinetics of TRPV3 ................................................................. 92
4.3. Functional characterization of TRPV3 sensitization ............................................. 96
4.4. Intracellular Ca^{2+} inhibits TRPV3 through CaM .................................................. 98
4.5. Identification of a CaM-binding site in the N-terminus of TRPV3 ..................... 99
4.6. Functional study of TRPV3RK ........................................................................ 100
4.7 TRPV3 is also inhibited by extracellular Ca^{2+} .................................................... 101
4.8 Characterization of extracellular Ca^{2+} block of TRPV3 ................................ 104
4.9 Identification of a high-affinity Ca^{2+}-binding site in the putative pore region of TRPV3 .......................................................... 105
4.10. Double mutations largely abolished Ca^{2+} inhibition of TRPV3 ............... 106
4.11. Summary ........................................................................................................ 108

Chapter 5 A point-mutation leading to constitutive TRPV3 channel activation is responsible for mouse hair-loss .............................................. 122

5.1 Introduction ........................................................................................................ 122
5.2 Expression of G573S and G573C mutants of TRPV3 kills host cells ...... 123
5.3 Ruthenium red partially rescued host cells expressing G573 mutants .... 124
5.4. Constitutive current of G573S and G573C mutants of TRPV3 expressed in *Xenopus* oocytes ............................................................. 125
5.5. Ligand-induced response of G573 mutants and wild type TRPV3 expressed in *Xenopus* oocytes ............................................................. 127
5.6 Heat-induced response of G573 mutants and wild type TRPV3 expressed in *Xenopus* oocytes ............................................................. 128
5.7 Expression of wild type TRPV3 partially rescued G573 mutant-expressing host cells ........................................................................ 130
5.8 Summary ........................................................................................................ 131
Chapter 6 Functional regulation of TRPC4 and TRPC5 ............................... 143

6.1 Introduction .............................................................................................................. 143
6.2 Stimulation of μ opioid receptor (μOR) specifically activates TRPC4/C5 but not other TRPCs ................................................................. 148
6.3 Other G\textsubscript{i/o}-coupled receptors also operate TRPC4 and TRPC5 .... 150
6.4 The excitatory effect of G\textsubscript{i/o} signaling pathway to TRPC4 and TRPC5 is PTX sensitive ................................................................. 152
6.5 Confirmation of the excitatory effects of G\textsubscript{i/o}-coupled receptors on TRPC4 and TRPC5 using whole-cell patch clamp recordings .......... 153
6.6 Synergistic effect between G\textsubscript{q/11} and G\textsubscript{i/o} signaling pathways for the activation of TRPC4 and TRPC5 ........................................... 153
6.7 Activation of TRPC4/C5 by G\textsubscript{i/o} signaling pathway does not occur through inhibiting cAMP/PKA pathway ................................................................. 156
6.8 PLC is required for the G\textsubscript{i/o}-induced TRPC4 and TRPC5 activation ...... 157
6.9 Intracellular Ca\textsuperscript{2+} is required for TRPC4 and TRPC5 activation induced by G\textsubscript{i/o} signaling ........................................................................ 160
6.10. PKG/cGMP, PKC, and PI3K are not involved in the coupling of G\textsubscript{i/o} pathway to TRPC4/C5 activation ......................................................... 161
6.11. Summary ............................................................................................................ 162

Chapter 7 Discussion and future perspective ......................................................... 181

BIBLIOGRAPHY ............................................................................................................. 194
LIST OF TABLES

TABLE 4.1 Solutions used for whole-cell recording experiments................111
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Phylogenetic tree of mammalian TRP channels</td>
<td>57</td>
</tr>
<tr>
<td>1.2</td>
<td>General architecture of TRP channels</td>
<td>58</td>
</tr>
<tr>
<td>1.3</td>
<td>Some important binding partners for TRP channels</td>
<td>59</td>
</tr>
<tr>
<td>3.1</td>
<td>AA potentiated TRPV3-mediated Ca(^{2+}) influx in both mouse keratinocytes and TRPV3-transfected HEK293 cells</td>
<td>82</td>
</tr>
<tr>
<td>3.2</td>
<td>AA specifically potentiated the currents of TRPV3 but not TRPV1 and TRPV2</td>
<td>83</td>
</tr>
<tr>
<td>3.3</td>
<td>Functional characterization of AA-potentiated TRPV3 current</td>
<td>84</td>
</tr>
<tr>
<td>3.4</td>
<td>AA increased the open probability of TRPV3</td>
<td>85</td>
</tr>
<tr>
<td>3.5</td>
<td>AA potentiated 2APB-evoked TRPV3 currents in Xenopus oocytes</td>
<td>86</td>
</tr>
<tr>
<td>3.6</td>
<td>AA dose-dependently potentiated 2APB-evoked TRPV3 currents in <em>Xenopus</em> oocytes</td>
<td>87</td>
</tr>
<tr>
<td>3.7</td>
<td>AA potentiated 2APB-evoked TRPV3 independent of kinases and its downstream metabolites</td>
<td>88</td>
</tr>
<tr>
<td>3.8</td>
<td>Multiple unsaturated fatty acids potentiated 2APB evoked TRPV3 currents in <em>Xenopus</em> oocytes</td>
<td>89</td>
</tr>
<tr>
<td>4.1</td>
<td>Intracellular Ca(^{2+}) buffering strength affected the activation kinetics of TRPV3 by 2APB</td>
<td>112</td>
</tr>
<tr>
<td>4.2</td>
<td>Intracellular Ca(^{2+}) buffering strength affected sensitization process of TRPV3 induced by repetitive 2APB stimulation</td>
<td>113</td>
</tr>
</tbody>
</table>
Figure 4.3 Changes of voltage dependence of TRPV3 during repetitive stimulations .................................................................114

Figure 4.4 The intracellular Ca²⁺ effect on TRPV3 was through CaM............115

Figure 4.5 Identification of a CaM binding site in the N-terminus of TRPV3.....116

Figure 4.6 Voltage dependence of TRPV3RK⁻ mutant in response to repetitive 2APB stimulations.................................................................117

Figure 4.7 Extracellular Ca²⁺ blocked TRPV3..............................................118

Figure 4.8 Extracellular Ca²⁺ dose-dependently inhibited TRPV3.................119

Figure 4.9 TRPV3D641N mutant abolished the extracellular Ca²⁺ high affinity inhibition.................................................................120

Figure 4.10 Effect of intracellular Ca²⁺/CaM binding on the activation of TRPV3D641N mutant.................................................................121

Figure 5.1 Expression of G573C and G573S mutants of mouse TRPV3 caused severe host cell death .........................................................136

Figure 5.2 The cell-killing effect of G573 mutants of TRPV3 was partially rescued by ruthenium red but not by lowering extracellular [Ca²⁺].................137

Figure 5.3 G573 mutants of TRPV3 were constitutively active when expressed in Xenopus oocytes.........................................................138

Figure 5.4 Agonists-induced responses of G573 mutants and wild type TRPV3 expressed in Xenopus oocytes.................................................139

Figure 5.5 Heat-induced responses of G573 mutants and wild type TRPV3 expressed in Xenopus oocytes.......................................................140

Figure 5.6 Temperature dependence of G573 mutants and wild type TRPV3 expressed in Xenopus oocytes.......................................................141

Figure 5.7 Partial rescue of G573 mutants expression by wild type TRPV3.....142

Figure 6.1 TRPC channels were functionally expressed using magnetic nanobeads-based transfection method.................................................166
Figure 6.2 TRPC4 and TRPC5 but not other TRPC channels were coupled to G\textsubscript{i/o} signaling pathway.................................................................167

Figure 6.3 DAMGO dose-dependently activated TRPC4 and TRPC5..............168

Figure 6.4 TRPC4 and TRPC5 were activated by G\textsubscript{i/o} signaling pathway........169

Figure 6.5 Effects of G\textsubscript{q/11} and G\textsubscript{i/o} signaling pathways for the activation of TRPC5.................................................................170

Figure 6.6 Activation of TRPC4/5 by G\textsubscript{i/o}-coupled μOR was PTX sensitive .................................................................171

Figure 6.7 G\textsubscript{i/o} signaling pathway activated TRPC4 and TRPC5 currents in whole-cell experiments.................................................................172

Figure 6.8 TRPC4\textsubscript{α} currents activated by G\textsubscript{q/11} and G\textsubscript{i/o} signaling pathways.................................................................173

Figure 6.9 TRPC5 currents activated by G\textsubscript{q/11} and G\textsubscript{i/o} signaling pathways.................................................................174

Figure 6.10 CCh-evoked TRPC4 currents in cells without or with G\textsubscript{i/o}-coupled M\textsubscript{2R} .................................................................175

Figure 6.11 Effect of forskolin on the activation of TRPC4 and TRPC5..............176

Figure 6.12 PLC was required for the excitatory effect of G\textsubscript{i/o} signaling pathway for TRPC4/C5.................................................................177

Figure 6.13 Activation of G\textsubscript{i/o} signaling pathway did not induce detectable Ca\textsuperscript{2+} release from ER stores.................................................................178

Figure 6.14 Activation of TRPC4/C5 by G\textsubscript{i/o} signaling pathway required intracellular Ca\textsuperscript{2+}.................................................................179

Figure 6.15 Excitatory effect of G\textsubscript{i/o} signaling pathway for TRPC4/C5 did not involve PI3K, PKC.................................................................180

Figure 7.1 Proposed model for TRPV3 during sensitization..............................193
ABBREVIATIONS

2-APB, 2-aminoethoxydiphenyl borate
4α-PDD, 4-phorbol 12,13-didecanoate
5HT₁AR, metabotropic serotonin receptor subtype 1
12-HPETE, 12-hydroperoxyeicosatetraenoic acid
17-ODYA, 17-octadecynoic acid
AA, arachidonic acid
AD, atopic dermatitis
ADPKD, autosomal dominant polycystic kidney disease
ADPR, ADP-ribose
B₂R, bradykinin receptor subtype 2
BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
BDNF, brain-derived neurotrophic factor
BSA, bovine serum albumin
cADPR, cyclic ADP-ribose
\([\text{Ca}^{2+}]\), concentration of free \(\text{Ca}^{2+}\)
\([\text{Ca}^{2+}]_i\), concentration of intracellular free \(\text{Ca}^{2+}\)
CaM, calmodulin
CaMKII, \(\text{Ca}^{2+}/\text{CaM}\)-dependent kinase II
CCE, capacitative calcium entry
CCh, carbacol
CDC, cinnamyl-3,4-dihydroxy-cyanocinnamate
Cdk5, cyclin-dependent kinase 5
cDNA, complementary DNA
CHO cell, Chinese hamster ovary cell
CIRB, calmodulin- and IP3R-binding
CNG channel, cyclic nucleotide-gated channel
CNS, central nerve system
CRAC, calcium-release activated calcium channel
cRNA, complementary RNA
D_{2}R, dopamine receptor subtype 2
DAG, diacylglycerol
DMEM, Dulbecco’s Modified Eagle’s Medium
DRG, dorsal root ganglion
ECS, extracellular solution
EGF, epithelial growth factor
EGFP, enhance green fluorescent protein
EGTA, ethylene glycol tetraacetic acid
ER, endoplasmic reticulum
ERG, electroretinogram
ETYA, 5,8,11,14-eicosatraynoic acid
FBS, fetal bovine serum
FLS cells, fibroblast-like synoviocytes
GI, gastrointestinal
G-V, conductance-voltage relationship
H₁R, histamine receptor subtype 1
HEDTA, Trisodium salt of N-(hydroxyethyl)-ethylenediaminetriacetic acid
HEK293 cell, human embryonic kidney 293 cell
IA, iodoacetamide
IP₃, inositol 1, 4, 5-trisphosphate
IP₃R, inositol 1, 4, 5-trisphosphate receptor
I-V, current-voltage relationship
KN-62, 4-[(2S)-2-[(5-isoquinolinylsulfonyl) methylamino]-3-oxo-3-(4-phenyl-1-piperazinyl) propyl] phenyl isoquinolinesulfonic acid ester
LTD, long-term depression
M₂R, muscarinic receptor subtype 2
M₃R, muscarinic receptor subtype 3
M₅R, muscarinic receptor subtype 5
MAP kinase, mitogen-activated protein kinase
MBP, maltose-binding protein
MTSEA, (2-aminoethyl)methanethiosulphonate
NADA, N-arachidonoyl dopamine
NDGA, nordihydroguaiaretic acid
NGF, nerve growth factor
NHERF, sodium hydrogen exchanger regulatory factor
NMDG, N-methyl-D-glucamine
NMM, N-methyl maleimide
NO, nitric oxide
OAG, 1-oleoyl-2-acetyl-sn-glycerol
OEA, oleoylethanolamide
P₂Y₅, metabotropic purinergic receptors
PBS, phosphate buffered saline
PCR, polymerase chain reaction
PDZ domain, postsynaptic density/discs-large/zonula occludens domain
PI₃K, phosphoinositide 3-kinases
PIP₂, Phosphatidylinositol (4, 5)-bisphosphate
PKC, protein kinase C
PKG, protein kinase G
PLA₂, phospholipase A₂
PLCβ, phospholipase C-β
PLD, phospholipase D
P-loop, pore loop
PTH, parathyroid hormone
PTX, pertussis toxin
PUFAs, poly-unsaturated fatty acids
Q₁₀, temperature coefficient over a 10°C range
RFU, relative fluorescence unit
RR, ruthenium red
S1-S6, transmembrane segment 1 to 6

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

siRNA, small interfering RNA

SPH, D-erythro-sphingosine

SOC, store-operated channel

TCEP, Tris (2-carboxyethyl) phosphine hydrochloride

TG, trigeminal ganglion

TRP, Transient Receptor Potential

TRPA, transient receptor potential ANKTM1

TRPC, transient receptor potential canonical

TRPM, transient receptor potential melastatin

TRPML, transient receptor potential mucolyptin

TRPN, transient receptor potential NOMP

TRPP, transient receptor potential polycystin

TRPV, transient receptor potential vanilloid

μOR, μ opioid receptor

$V_{1/2}$, the voltage for half-maximal activation

VDF, voltage-dependent fraction

VON, vomeronasal neurons

VR1, capsaicin receptor
CHAPTER 1

INTRODUCTION

1.1 A trip to TRP: historical overview

Back in 1969, Cosens and Manning firstly isolated and characterized a *Drosophila* mutant allele with abnormal electroretinogram (ERG), which was due to a fully recessive gene mutation on the third chromosome (Cosens & Manning, 1969). Minke and his colleagues designated this mutant *Transient Receptor Potential* (TRP) because the mutation caused a fast decay of ERG during strong light stimulation compared to the sustained plateau ERG in wild type (Minke et al., 1975). It took more than two decades for investigators to eventually clone the *Drosophila* trp gene (Montell & Rubin, 1989). The cloned trp gene encodes a 1275 amino acid protein and is predominantly expressed in the rhabdomeric membranes of the photoreceptor cells of the insects. Later on, researchers found that the TRP channel was a nonselective cation channel that formed part of the light-induced conductance in *Drosophila* eyes (Hardie & Minke, 1992; Xu et al., 1997). A trp-like gene, trpl, was cloned from *Drosophila* in 1992 (Phillips et al., 1992), and it encodes a homologous channel called TRPL (Niemeyer et al., 1996). TRP and TRPL channels account for the complete light conductance of
the Drosophila eye (Niemeyer et al., 1996). In the meantime, two independent groups cloned the first mammalian TRP homolog, TRP1, in 1995 (Wes et al., 1995; Zhu et al., 1995). Six other mammalian homologs were subsequently cloned in full-length in following years based on sequence homology (Zhu et al., 1996; Okada et al., 1998; Okada et al., 1999; Philipp et al., 1996; Philipp et al., 1998). Functional characterizations of these mammalian TRP channels in native cells and heterologous expression systems suggested that several TRP channels were involved in capacitative calcium entry (CCE) pathway, which made some groups believe TRP channels were the molecular basis of calcium-release activated calcium (CRAC) channels.

In 1997, David Julius and colleagues cloned capsaicin receptor (VR1, TRPV1) using an expression cloning strategy (Caterina et al., 1997). It turned out VR1 is also a heat-activated non-selective cation channel with a temperature activation threshold of 42ºC. Surprisingly, this receptor showed a homology to TRP channels; therefore it is now termed TRPV1. Three other homologous genes, TRPV2-4, were cloned in the following five years based on sequence homology and functional studies showed that they are also heat activated, but with different temperature threshold than TRPV1. In 2002, a cold-activated TRP channel, TRPM8, was cloned independently by Julius’ and Patapoutian’s laboratories. Opposite to TRPV1, TRPM8 was activated by both cooling reagent menthol and low temperatures.
A unified nomenclature was proposed in 2002 for the TRP superfamily of ion channels (Montell et al., 2002). Based on the DNA sequence alignment and functional relationships, the mammalian TRP superfamily was divided into six sub-families: canonical TRPs (TRPC) are those that show highest homology to the original *Drosophila* TRP protein; vanilloid TRPs (TRPV) were designated based on the founding member vanilloid receptor 1 (VR1, also known as capsaicin receptor or TRPV1); melastatin TRPs (TRPM) are named after the founding member Melastatin (TRPM1). Ankyrin TRP (TRPA1) represents the single mammalian ANK-TRP; polycystin TRPs (TRPP) were named after polycystic kidney disease gene, PKD2; mucolipin TRPs (TRPML) are homologues of the gene responsible for mucoliposis type IV. In addition, TRPNs are only found in invertebrates. At least 28 rodent TRP genes, 27 human TRP genes (TRPC2 in human is a pseudogene), 17 *C. elegans* TRP genes, and 12 *Drosophila* TRP genes are present according current database collections (Nilius, 2006). The evolutionary relationships of TRP proteins (mainly mammalian TRPs) are shown in figure 1.1.

TRPV1, V2, V3, V4, M8, and A1 are also called thermo-TRPs because of their roles in temperature sensation. Due to the obviously important sensory functions and specific pharmacological tools available, the thermo-TRPs (especially TRPV1, TRPM8 and TRPA1) have received the most attentions in the past ten years among the TRP superfamily. Much has been discovered for these TRP channels including the physiological roles and activation and regulation
mechanisms. The results from these studies also provide some useful clues on the function and regulation of other TRP channels. Currently, thermo-TRPs are still a hot research topic, but more and more efforts have been put on the studies of other TRP channels.

1.2 General architecture of TRP channels
Although it was originally proposed that TRP channels had 8 transmembrane segments based on the hydrophobicity profile (Montell & Rubin, 1989), it turned out that the third and the seventh predicted hydrophobic domains are not really transmembrane, giving rise to a six transmembrane domain topology (figure 1.2 A). Similar to other six transmembrane domain ion channels, such as voltage-gated potassium (K+) channels and cyclic nucleotide-gated (CNG) channels, the basic architecture of TRP channels is formed by four subunits with each subunit containing six transmembrane segments (S1-S6) (figure 1.2 C and D), and the loop between S5 and S6 transmembrane segments forms the pore loop (P-loop) (Nilius, 2006) (figure 1.2 B). Some TRP subunits are able to interact with other types of TRP subunits to form hetero-tetramers, such as TRPC1/C4, TRPC1/C5, TRPV5/V6, TRPM6/M7 (Strübing et al., 2001; Strübing et al., 2003; Hoenderop et al., 2003; Li et al., 2006); but in most known cases when heteromultimerization was studied, it only occurs within the same subfamily.

The S4 segment of a voltage-gated K+ channel has multiple positively-charged lysine residues, which form the voltage sensor (Long et al., 2005). However, TRP
channels lack this kind of sensor in their S4 segments, therefore, most TRP channels are not voltage-gated, or more precisely, not activated by membrane depolarization in a manner similar to classical voltage-gated $K^+$ channels. However, as will be described later, voltage sensitivity is present for some TRP channels.

Some common structural features exist among different TRP subfamilies (figure 1.3). First, there is a conserved TRP domain downstream from the S6 segment of all TRPC, TRPV, and TRPN members. It has been shown that this TRP domain might be involved in phosphatidylinositol (4, 5)-bisphosphate (PIP$_2$) binding for some TRP channels (Rohacs et al., 2005), but the precise function of TRP domain remains unclear for most other TRP channels. Second, ankyrin-like repeats are another common feature across several TRP subfamilies: with TRPC subfamily containing four repeats, TRPV subfamily containing three repeats, and TRPA and TRPN subfamilies containing more than ten. The ankyrin domain is a 30-34 amino acid segment thought to be involved in protein-protein interactions (Li et al., 2006). At least one study has indicated that the ankyrin-like repeats of TRP channels are involved in the multimerization and trafficking of these channels (Arniges et al., 2006). Third, similar to other calcium permeable ion channels, such as voltage-gated $Ca^{2+}$ channels and CNG channels, many TRP channels are regulated by calcium ions via calmodulin (CaM). CaM-binding site(s) is present in many, if not all, TRP channels, and the regulation of TRP channels by CaM and other $Ca^{2+}$-binding proteins can be rather complicated (Zhu, 2005).
The general channel topology for TRP channels and some important structural domains are shown in figure 1.3.

1.3 Diverse functions of TRP channels

1.3.1 Physiological functions of TRPC channels

The founding member of TRP channel superfamily is the *Drosophila* TRP. In *Drosophila*, TRP channels are restricted in *Drosophila* eyes and they mediate light-induced conductance. Briefly, photons hit the molecule of *Drosophila* retinal and activate G protein-coupled receptor, rodopsin, which in turn activates phospholipase C-β (PLCβ). The *Drosophila* TRP channels are opened via downstream event(s) of the PLCβ pathway and they mediate Na⁺ and Ca²⁺ influx. TRP-deficient flies are blinded by intense light because of the damaged Ca²⁺ entry (Minke & Parnas, 2006; Montell, 2005). Thus, the most important physiological function of TRP channels in insects is to provide a Ca²⁺ entry pathway and a depolarizing force.

In the mammalian TRP superfamily, seven TRPC channels have the highest sequence homology to the *Drosophila* TRP. Based on the sequence homology and functional similarity, the TRPC subfamily is further divided into 4 groups: TRPC1, TRPC2, TRPC3/6/7, and TRPC4/5. Because of the sequence similarity to the *Drosophila* TRP, it is reasonable to speculate that mammalian TRPC channels might play a similar role in PLCβ-mediated cell signaling. Indeed, in multiple types of cells, including endothelial cells (Ahmmed et al., 2004; Brough
et al., 2001) and smooth muscle cells (Beech et al., 2004), endogenous TRPC1 (which was the first cloned mammalian TRP based on homology screening) has been shown to be important for Ca$^{2+}$ entry activated through receptors stimulation. XTRPC1, a *Xenopus* homolog of mammalian TRPC1, is shown to mediate Ca$^{2+}$ influx that is important for axon growth cone turning response to multiple secreted signaling molecules such as netrin and brain-derived neurotrophic factor (BDNF) (Shim et al., 2005).

TRPC2 is a pseudogene in human (Wes et al., 2001), but rodent TRPC2 is highly expressed in the vomeronasal organ (Hofmann et al., 2000). TRPC2 plays an essential role in pheromone detection of rodents as knocking-out TRPC2 in mice greatly affected gender discrimination and male-male aggression behaviors (Stowers et al., 2002). Native TRPC2 channels were shown to be activated by diacylglycerol (DAG), a downstream signaling molecule of G protein-coupled pheromone receptors. The opening of the TRPC2 channel causes Na$^+$ and Ca$^{2+}$ influx, providing a depolarizing pulse for vomeronasal neurons (VON) (Lucas et al., 2003). Thus, TRPC2 in rodent vomeronasal organs converts the chemical signal (pheromones) to electrical signal. Although the function of vomeronasal organs in rodents is clearly important for their sexual reproduction, the existence and function of a similar system in humans is still under debate (Bigiani et al., 2005). Interestingly, the loss of TRPC2 appears to be associated with the development of tri-color vision in high primates, suggesting an evolutionary
change from pheromone to vision-based mate selection and the importance of
TRPC2 in the former but not the latter reproductive behavior.

TRPC3, TRPC6 and TRPC7 share about 75% sequence identity (Clapham,
2003). TRPC3 is highly expressed in brain (Zhu et al., 1996; Li et al., 1999), and
there is evidence that TRPC3 is important for BDNF-mediated growth cone
guidance in cerebellar granule neurons (Li et al., 2005). BDNF is a chemo-
attractive signal for granule neuron growth cones, and the application of BDNF to
cultured granule neurons induces intracellular Ca\(^{2+}\) elevation. TRPC3 channel
was shown to mediate the Ca\(^{2+}\) entry induced by BDNF in granule neurons (Li et
al., 2005) and pontine neurons (Li et al., 1999). TRPC6 is highly expressed in
brain and lung, and TRPC7 is highly expressed in heart and lung
(Venkatachalam & Montell, 2007). TRPC6 has been shown to form the Ca\(^{2+}\)-
permeable channel activated by \(\alpha_1\)-adrenoreceptors in rabbit portal vein smooth
muscle (Inoue et al., 2001), indicating a vasoregulation role of TRPC6. In a
recent study, it was found that TRPC3 and TRPC6 mediated BDNF-induced Ca\(^{2+}\)
elevation in cerebellar granule neurons and were important for promoting
neuronal survival (Jia et al., 2007). The physiological role of TRPC7 is still not
very clear at this stage, although there are a few in vitro experiments indicating
that TRPC7 might be involved in heart failure induced by angiotensin II
overexpression during hypertrophy (Arata et al., 1999; Satoh et al., 2007).
TRPC4 and TRPC5 share 64% sequence identity and a large tissue distribution similarity (Ramsey et al., 2006). At least two characterized TRPC4 splicing variants exist in mammals: TRPC4\(\alpha\) and TRPC4\(\beta\), and it has been reported that TRPC4\(\alpha\) functioned as a dominant-negative regulator of TRPC4\(\beta\) (Schaefer et al., 2002). Strong evidence with TRPC4 knockout mice has proven that TRPC4 provided a \(\text{Ca}^{2+}\) entry pathway in native vascular endothelial cells and was critical for vasorelaxation (Freichel et al., 2001). TRPC5 is highly expressed in CA1 and CA3 regions of rodent hippocampus. It has been shown that TRPC5 is a regulator of hippocampal neurite length and growth cone morphology through unknown mechanism (Greka et al., 2003).

Although TRPC subfamily is the first cloned group of TRP channels, the complete physiological roles of TRPC channels in many tissues are still largely unclear. More detailed investigation of their physiological roles and activation mechanism is hindered by the lack of specific pharmacological tools. No established specific agonist has been found for any TRPC channel. All known TRPC channel blockers currently in use are non-specific.

1.3.2 Physiological functions of TRPV channels

It was the TRPV1 that attracts most attention in the TRP field. TRPV1 was first cloned as a capsaicin and heat receptor. More importantly, it is involved in the pain sensation (Caterina et al., 1997). TRPV1 has a specific naturally-occurring agonist: capsaicin (noxious ingredient from "hot" chili pepper), which has greatly
facilitated the research on TRPV1 (Caterina et al., 1997). To date, 4 members of the TRPV subfamily have been shown to be activated by heat. They are TRPV1-4. TRPV5 and TRPV6 are highly Ca$^{2+}$ selective channels involved in Ca$^{2+}$ transport in kidney and intestine. There is no reported data suggesting that they are heat sensitive (Mensenkamp et al., 2006).

The function of TRPV1 is probably the most extensively investigated in the TRP superfamily. TRPV1 was cloned from rat dorsal root ganglion (DRG) neurons. It is most highly expressed in DRG and trigeminal ganglion (TG) neurons (Caterina et al., 1997), but found in other regions such as skin keratinocyte (Inoue et al., 2002), mast cell (Stander et al., 2004), and gastrointestinal (GI) track (Avelino et al., 2002). The best known function of TRPV1 is that it is the primary receptor of capsaicin, the pungent ingredient of the very popular food - chili pepper (Caterina & Julius, 2001); besides, it is also a very important mediator for pain sensation and tissue injury-induced thermal hyperalgesia (Caterina et al., 2000). Because TRPV1 has a large pore and it is quite specifically expressed in nociceptor (pain-sensing) neurons but not motor neurons, researchers have proposed an ingenious way to achieve pain-specific local anesthesia (Binshtok et al., 2007). Basically, by co-application of TRPV1 agonist capsaicin and a hydrophilic membrane non-permeable sodium (Na$^+$) channel blocker, QX-314, the opening of TRPV1 channels allowed QX-314 to go through the large TRPV1 pore and block voltage-gated Na$^+$ channels from cytosolic side. This treatment blocked the excitability of pain-sensing neurons, achieving specific anesthesia (Binshtok et al.,
In addition to functioning as a molecular pain- and thermo-sensor in peripheral nerve system, TRPV1 might also be involved in the detection of bladder filling (Daly et al., 2007). In the central nerve system (CNS), TRPV1 is presumably not involved in the temperature and inflammation sensing due to the relatively stable environment. TRPV1 in hippocampal interneurons has been shown to be critical for the induction of long-term depression (LTD) (Gibson et al., 2007).

TRPV2 is expressed in medium to large diameter DRG neurons (Caterina et al., 1999) and smooth muscle cells (Muraki, et al., 2003), but the physiological role of TRPV2 is poorly understood compared with its close homolog TRPV1. It has been shown that TRPV2 is a key regulator of skeletal and cardiac myocyte degeneration (Iwata et al., 2003).

TRPV3 is expressed in skin keratinocytes (Peier et al., 2002b), tongue epithelium (Xu et al., 2006), brain, spinal cord, DRG and testis (Xu et al., 2002; Smith et al., 2002). The most important function of TRPV3 is thought to detect environmental temperature changes in the mild range. In TRPV3 knockout mice, the thermo-sensation was impaired compared with wild type animals (Moqrich et al., 2005). TRPV3 might also be the initial detector of some natural compounds from plants, such as camphor (Moqrich et al., 2005), oregano, thyme and some clove-derived flavors and skin sensitizers (Xu et al., 2006).
TRPV4 is widely distributed in many tissues, including skin (Chung et al., 2003), kidney, lung, spleen, fat, and CNS (Liedtke et al., 2000). The best known function of TRPV4 is to sense hypotonicity stress because it is activated by subtle changes of osmolarity in the physiological range (Liedtke et al., 2000). Similar to TRPV3, TRPV4 in skin keratinocytes may also be involved in the detection of mildly warm temperature (Chung et al., 2003).

TRPV5 and TRPV6 were originally cloned as ECaC1 and ECaC2, respectively. They shared about 75% sequence homology (Hoenderop et al., 2001). TRPV5 and TRPV6 are distinct from other TRPV members in that they are not temperature sensitive and they have a high Ca\textsuperscript{2+} selectivity (Hoenderop et al., 2001). TRPV5 and TRPV6 are highly expressed in kidney and intestine (Hoenderop et al., 2000; Hoenderop et al., 2001), where they play essential roles in Ca\textsuperscript{2+} uptake (Hoenderop et al., 2002).

1.3.3 Physiological functions of TRPM channels

Based on sequence alignment, TRPM subfamily is divided into 4 groups: TRPM1/3, TRPM2/8, TRPM4/5, and TRPM6/7. Due to the facts that multiple specific ligands are available and it is the first known cold-activated ion channel, TRPM8 has received most extensive studies among TRPM subfamily.

TRPM1, also called melastatin, is the founding member of the TRPM subfamily. TRPM1 was cloned in a search for putative genes responsible for an aggressive
phenotype of B16 murine melanoma line (Duncan et al., 1998). TRPM1 expression level was significantly reduced in highly metastatic B16 variants, indicating that TRPM1 might function as a tumor suppressor (Duncan et al., 1998). TRPM3 is mainly expressed in kidney, and to a less extent in brain (Grimm et al., 2003). However, the physiological roles of TRPM3 are still poorly understood.

TRPM2 is highly expressed in brain, lung, and spleen (Hara et al., 2002). A very interesting feature of TRPM2 is that it has an intrinsic ADPR pyrophosphatase domain within its C-terminus, which catalyses the removal of the terminal ribose-5-phosphate moiety of ADP–ribose (Perraud et al., 2001). Because TRPM2 can be activated by reactive oxygen species such as H$_2$O$_2$ (Hara et al., 2002), it may function as a cellular redox sensor. A recent study showed that TRPM2 in pancreatic islets was greatly potentiated by the co-application of body temperature heat and intracellular cyclic ADP-ribose (cADPR). This activation increased intracellular Ca$^{2+}$ concentration of islets and promoted insulin secretion (Togashi et al., 2006), suggesting the involvement of TRPM2 in the regulation of insulin secretion. TRPM8 is highly expressed in DRG neurons (Peier et al., 2002a), TG neurons (Peier et al., 2002a; McKemy et al., 2002), and prostate (Tsavaler et al., 2001). Based on the experiments using knockout mice performed in three independent laboratories, the major physiological role of TRPM8 is to function as the primary detector of environmental cold (Bautista et al., 2007; Dhaka et al., 2007; Colburn et al., 2007).
TRPM4 is expressed in multiple tissues including heart, placenta, kidney, liver, pancreas, and spleen (Launay et al., 2002), while TRPM5 is expressed in tongue, stomach, and small intestine (Pérez et al., 2002). Both TRPM4 and TRPM5 are directly activated by the elevation of intracellular \([\text{Ca}^{2+}]\) (Launay et al., 2002; Liu & Liman, 2003); therefore, they could potentially act as an intracellular \(\text{Ca}^{2+}\) sensor. It has been established that TRPM5 is required for the transduction of sweet, bitter and umami taste sensation (Zhang et al., 2003). In TRPM5 deficient animals, the taste reception of sweet, bitter, and umami taste, but not that of salty or sour taste, were diminished (Zhang et al., 2003).

TRPM6 is highly expressed in kidney, small intestine, and testis (Walder et al., 2002), whereas TRPM7 is expressed in kidney, heart, liver, spleen, and brain (Runnels et al., 2001). Both TRPM6 and TRPM7 have an atypical serine/threonine kinase domain in their C-termini, which makes them together with TRMP2 uniquely function as both an ion channel and a kinase (Nadler et al., 2001; Montell, 2003). TRPM6 and TRPM7 are both activated by a reduction of intracellular Mg\(^{2+}\) and Mg-ATP concentrations (Voets et al., 2004). The opening of both channels induces Mg\(^{2+}\) influx, thus providing a feedback mechanism to regulate intracellular Mg\(^{2+}\) homeostasis. Mutations of TRMP6 have been associated with hypomagnesemia disease in human (Schlingmann et al., 2002), and a targeted deletion of TRPM7 in DT-40 cell line caused a growth arrest and eventually cell death due to intracellular Mg\(^{2+}\) deficiency (Schmitz et al., 2003).
Therefore, TRPM6 and TRPM7 play central roles in Mg\(^{2+}\) homeostasis by mediating Mg\(^{2+}\) uptake in kidney, small intestine, and other tissues (Montell, 2003).

1.3.4 Physiological functions of TRPA1

There is only one mammalian TRPA member, TRPA1. TRPA1 is highly expressed in sensory neurons, such as DRG neurons and TG neurons (Story et al., 2003), and inner ear hair cells (Corey et al., 2004). In the original cloning paper, TRPA1 expressed in Chinese hamster ovary (CHO) was activated by a noxious cooling temperature of <17ºC, making it the second cold-sensitive TRP channel (Story et al., 2003). Besides, it co-localized with the noxious heat-activated TRPV1 in DGR sensory neurons, implying a universal role of TRP channels in mammalian temperature sensing (Story et al., 2002). However, follow-up experiments in some other laboratories could not faithfully reproduce the cold activation effect of TRPA1 (Jordt et al., 2004), so the temperature sensitivity of TRPA1 is still under debate. On the other hand, it has been shown that TRPA1 is the primary receptor for several pungent natural compounds including mustard oil (the pungent ingredient of wasabi), and illicin (the pungent ingredient from raw garlic) (Macpherson et al., 2004). The sensitivities of TRPA1 to these compounds and colocalization with TRPV1 led to the proposal that TRPA1, similar to TRPV1, also played an important role in pain sensation (Jordt et al., 2004). TRPA1 is highly expressed in hair cells of rodent inner ear, and the knockdown of TRPA1 protein level using RNA interference (RNAi) damaged the
hearing capability, indicating the essential role of TRPA1 for hearing (Corey et al., 2004) (this conclusion was disputed later).

However, the most convincing evidence regarding the physiological roles of TRPA1 came from TRPA1 knockout studies. Two independent groups have generated TRPA1 knockout mice using slightly different strategies (Bautista et al., 2006; Kwan et al., 2006). Both studies have found that TRPA1 knockout mice displayed normal hearing capability, thus disputing the essential role of TRPA1 in the hearing transduction of inner ear hair cell. The major discrepancy between these two studies is the sensitivity of TRPA1 to cold temperature. Since TRPM8 has been firmly established as a major environmental cold detector by three different laboratories (Bautista et al., 2007; Dhaka et al., 2007; Colburn et al., 2007), TRPA1 might not be a major player for environmental cold sensation. In contrast, multiple environmental irritants, including acrolein and 2-pentenal (highly toxic aldehydes present in tear gas and vehicle exhaust), and pungent ingredients from plant sources, such as mustard oil and allicin all activated TRPA1 in a subset of sensory neurons and induced inflammation and pain sensation (Jordt et al., 2004; Bautista et al., 2007;). Thus, TRPA1 might play an important role in detecting environmental and endogenous proalgesic agents (Bautista et al., 2007).

1.3.5 Physiological functions of TRPP channels
The discovery of TRPP channels was due to the genetic analysis of autosomal dominant polycystic kidney disease (ADPKD) (Geng et al., 1996). Mutations of two polycystic kidney disease genes, PKD1 and PKD2 (now termed TRPP1 and TRPP2, respectively), are responsible for ADPKD (Geng et al., 1996). The product of PKD1, polycystin (or TRPP1), is a large transmembrane protein containing 4302 amino acids and has a very long (about 2500 amino acids) extracellular N-terminus (Hughes et al., 1995). Based on the hydrophobicity profile, it was proposed that TRPP1 had eleven transmembrane domains (Hughes et al., 1995). The product of PKD2, polycystin 2 (or TRPP2), is a 110 kD transmembrane protein sharing a common topology with TRP channels: six transmembrane segments with both N- and C- termini being intracellular (Cai et al., 1999). It has been shown that TRPP2 is primarily located in endoplasmic reticulum (ER) and Golgi apparatus, and TRPP1 is required to recruit TRPP2 into plasma membrane to function as a Ca$^{2+}$ permeable channel (Hanaoka et al., 2000). Because TRPP1 and TRPP1-like proteins are not TRP channels per se, only TRPP2 and its homologues will be discussed here.

There are three TRPP members: TRPP2, TRPP3, and TRPP5, but little is known about TRPP3 and TRPP5. TRPP2 is widely expressed in multiple tissues, including kidney, liver, skeletal muscle, lung, heart, and placenta (Nomura et al., 1998). TRPP2 mutations are responsible for about 15% of all ADPKDs (Tsiokas et al., 2007). TRPP2 is a Ca$^{2+}$ permeable cation channel both on the plasma (Nauli et al., 2003) and intracellular membranes (Koulen et al., 2002). However,
the detailed molecular mechanism by which the mutations of TRPP2 lead to ADPKD is still unclear.

1.3.6 Physiological functions of TRPN channels

TRPN family is named after the founding member NOMPC in *Drosophila* (Walker et al., 2000). A single TRPN member channel exists in *Drosophila, C. elegans, and zebra fish*, (termed dNOMPC, cNOMPC, and zTRPN1, respectively), but there is no mammalian TRPN gene (Venkatachalam et al., 2007). All TRPN channels are known to be mechanically gated, indicating that they might play important roles in sensing osmolarity change and local motion.

1.3.7 Physiological functions of TRPML channels

TRPML subfamily contains three mammalian members: TRPML1-3. TRPML1 (also called mucolipin) was cloned from the genetic analysis of a lysosomal storage disorder neuro-degeneration disease-mucolipidosis type IV (Sun et al., 2000). Both TRPML1 and TRPML2 reside in the lysosome membrane and function as Ca^{2+} permeable intracellular channels (Pedersen et al., 2005). The lysosomal localization of TRPML3 might require TRPML1 and TRPML2 (Venkatachalam et al., 2006). Functionwise, TRPML1 and TRPML2 are important for maintaining lysosomal Ca^{2+} level and normal lysosome formation (Pedersen et al., 2005), while the mutation of TRPML3 in mouse inner ear hair cell causes varitint-waddler (Va) deafness, indicating that TRPML3 might be the transduction channel in inner ear (Atiba-Davies & Noben-Trauth, 2007).
1.3.8 A generalized function for TRP channel superfamily

From the discussion above, we can see that the TRP channel superfamily members have rather diversified functions: from pheromone detection to taste transduction; from light transduction to sensing mechanic stimulation; from temperature sensing to pain sensing; from Ca\(^{2+}\) entry pathway to Mg\(^{2+}\) uptake pathway, and etc. In general, TRP channels are involved in detecting the chemical and physical stimuli from both intracellular and extracellular sides-functioning as a cellular sensor (Clapham, 2003). The mechanisms for TRP channel to play an important sensor role involve generating a cation entry (most likely Ca\(^{2+}\) entry, and to a less extent Mg\(^{2+}\) entry) pathway and depolarizing force. However, the opening of many other types of cation channels, such as voltage-gated Ca\(^{2+}\) channels, CNG channels, ligand-gated N-type acetylcholine receptor, and NMDA receptor, can also induce the same results-Ca\(^{2+}\) entry and depolarization. The key for TRP channels is that they are activated and regulated by many more factors than other channels (discussed in detail below).

1.4 Activation and regulation mechanisms of TRP channels

TRP channels are cellular sensors (Clapham, 2003), which means that they must detect multiple intracellular and extracellular molecular cues. Indeed, the activation and regulation mechanisms of TRP channels are very complex, and there is a general agreement in the field that most if not all TRP channels are polymodal sensors (Ramsey et al., 2006).
1.4.1 Activation and regulation of TRPC channels

1.4.1.1 Information learnt from *Drosophila* TRPs

TRPC subfamily channels share the highest homology with the *Drosophila* TRP channels and their activation also involves a common signaling pathway coupled to PLC (Venkatachalam et al., 2007). PLC plays an essential role for phototransduction of *Drosophila*. A nearly null PLC mutant norpA was not sensitive to supersaturating intensity light stimulation (Pak, 1995). Interestingly, a PLC inhibitor U-73122, but not its inactive analog U-73343, inhibited TRPL channel expressed in Sf-9 cells (Estacion et al., 2001; Minke & Parnas, 2006), indicating that TRP and TRPL channels might be the targets of PLC in *Drosophila* phototransduction. *In vivo* studies in *Drosophila* have shown that the activation of TRP and TRPL requires concerted actions of intracellular [Ca\(^{2+}\)] elevation and DAG production (Minke & Parnas, 2006). Since both signaling events are downstream of PLC\(\beta\) activation, taken together, TRP and TRPL channels are regulated by PLC\(\beta\) and function as the photo-transduction channels in *Drosophila*.

Another important feature of *Drosophila* TRP channels is the formation of the multi-molecule signaling complex- termed “signalplex” (Montell, 2005). With the help of a PDZ (postsynaptic density/disclarge/zonula occludens) domain-containing scaffolding protein-INAD, the photo-transduction signalplex of *Drosophila* contains at least eight protein molecules: TRP channel, TRPL channel, rhodopsin, CaM, protein kinase C (PKC), PLC, INAD, and actin-binding
protein NINAC (Montell, 2005). It is thought that the formation of this signalplex can greatly facilitate the regulation efficiency of TRP channels by these proteins.

1.4.1.2 Proteins that interact with TRPC channels

Similar to *Drosophila* TRPs, mammalian TRPC channels also have many binding partners. One of the interesting ones is CaM. All TRPC channels and *Drosophila* TRP and TRPL contain a conserved CaM-binding site (CIRB site) in their C-termini. Strikingly, this CaM-binding site also binds to inositol 1, 4, 5-trisphosphate receptors (IP3R) (Tang et al., 2001). It has been shown that IP3R and CaM bind to CIRB site in a competitive way-IP3R bound to the CIRB site and activated TRPC3 channel by replacing the inhibitory CaM (Zhang et al., 2001).

Besides CIRB site, some TRPC proteins have multiple other CaM-binding sites; for example, TRPC5 has one CaM-binding site on the C-terminus and another one on the N-terminus (Zhu, 2005). Functionally, different CaM-binding sites might play distinct roles. For instance, CaM-binding to the CIRB site of TRPC5 might be inhibitory, whereas the binding on the distant C-terminus is facilitatory for the activation of TRPC5 (Ordaz et al., 2005). Given that many TRPC channels have multiple CaM binding sites (Zhu, 2005), the regulation of TRPC channels by CaM can be rather complicated. TRPC4 and TRPC5 also bind to a PDZ-domain containing scaffolding protein-sodium hydrogen exchanger regulatory factor (NHERF), which links TRPC4 and TRPC5 to PLCβ and might work as a functional homolog of INAD in *Drosophila* eyes (Tang et al., 2000).
1.4.1.3 The activation and regulation of TRPC1

There are plenty of reports that TRPC1 forms a store-operated channel (SOC) (Pederson et al., 2005). In this regard, it is the depletion of ER Ca\(^{2+}\) store \textit{per se} but not the released Ca\(^{2+}\) that leads to the activation of TRPC1. TRPC1 expressed in CHO and Sf9 cells was shown to be activated by intracellular infusion of IP\(_3\) and thapsigargin (both compounds are supposed to deplete the ER Ca\(^{2+}\) store) (Zitt et al., 1996). The working mechanism for the activation of TRPC1 by ER Ca\(^{2+}\) store depletion could be due to a physical interaction between TRPC1 in the plasma membrane and IP\(_3\)R in the ER membrane (Yuan et al., 2003). However, these data should be interpreted with caution. In the experiments by Zitt et al., IP\(_3\) and thapsigargin not only depleted ER Ca\(^{2+}\) store but also increased cytosolic free Ca\(^{2+}\) concentration. Therefore, the activation of TRPC1 could be due to the increase of Ca\(^{2+}\) concentration, but not the depletion of ER store \textit{per se}.

Moreover, there is still some functional discrepancy between heterologously expressed TRPC1 and native SOC: first, TRPC1 is a nonselective cation channel while many SOCs recorded are highly Ca\(^{2+}\)-selective (Zitt et al., 1996; Parekh & Putney, 2005); second, most biophysical parameters of TRPC1 obtained in heterologous expression systems are not the same as those from Ca\(^{2+}\) release-activated Ca\(^{2+}\) channel (\(I_{\text{crac}}\)) (the best characterized SOC) (Parekh & Putney, 2005) in native systems; third, a systematic RNAi-based approach has been employed to knockdown endogenous TRPC1 channels in \textit{Drosophila} S2 cells. It
turned out that the reduction of TRPC1 protein level by RNAi had no effect on SOC (Liou et al., 2005; Roos et al., 2005). Therefore, it remains questionable whether TRPC1 is an essential component of native SOCs.

Some evidence also indicates that TRPC1 is activated by mechanical stretch. When TRPC1 was expressed in either *Xenopus* oocytes or CHO cells, a huge increase of stretch-activated non-selective cation current was detected, which was not present when control DNA construct was transfected (Maroto et al., 2005). Importantly, injection of TRPC1 antisense RNA abolished the endogenous stretch-activated current, suggesting that TRPC1 was the underlying molecule for the endogenous stretch-activated current (Maroto et al., 2005). TRPC1 was also shown to regulate cell volume by functioning as a stretch-activated ion channel. Knockdown of endogenous TRPC1 by small interfering RNA (siRNA) in H4-IIE liver cells led to a greatly increased cell volume when cells were challenged with hypotonic solutions (Chen & Barritt). However, the mechanism governing the stretch sensitivity of TRPC1 is unclear.

1.4.1.4 The activation and regulation of TRPC2

In rodent vomeronasal organs, two types of G protein-coupled pheromone receptors exist: V1Rs are expressed in the apical zone of VON, and V2Rs are expressed in the basal zone of VON (Dulac & Axel, 1995; Herrada & Dulac, 1997; Matsunami & Buck, 1997). Importantly, V1Rs co-localize with Gαi2, and V2Rs co-localize with Gαo in two subsets of VONs (Matsunami & Buck, 1997). The
activation of V1Rs and V2Rs by pheromones generates GTP-bound active \( \text{G}_{\alpha i/o} \) and \( \text{G}_{\beta\gamma} \), which in turn activate PLC signaling pathway and produce IP\(_3\) and DAG. DAG has been shown to directly activate native TRPC2 current in the dendrites of VON, therefore, TRPC2 functions as a receptor-operated channel in the vomeronasal organ (Lucas et al., 2003).

TRPC2 has been reported to bind to CaM (Tang et al., 2001), enkurin (Sutton et al. 2004), and Junctate (Treves et al. 2004; Stamboulian et al. 2005), but the functional significance for these interactions is unknown.

1.4.1.5 The activation and regulation of TRPC3, C6, C7

This group of TRPC channels is unique in that they are directly activated by an important lipid second messenger DAG without involvement of downstream signaling, such as PKC activation (Hofmann et al., 1999). However, DAG cannot account for the activation of TRPC3/6/7 in all cases. For instance, in freshly isolated rat pontine neurons, TRPC3 was highly expressed and co-localized with the BDNF receptor, TrkB. BDNF application activated a native TRPC3-like current which was blocked by a TRPC3-specific antibody but not a control antibody. Surprisingly, membrane permeable DAG analog OAG did not induce this TRPC3-like current in pontine neurons (Li et al., 1999), which was inconsistent with the experiments done with TRPC3 heterologously expressed in CHO cells where OAG alone directly activated TRPC3 current in both whole-cell and inside-out configurations (Hofmann et al., 1999). It was also observed that
this TRPC3-like current was blocked by PLC inhibitor U73122 and IP3R inhibitor Xestospongin-C (Li et al., 1999). Thus, it appears that the activation of endogenous TRPC3 may not involve DAG, although PLC is still essential. There are still a lot of controversies regarding the molecular mechanisms of the activation of TRPC3/6/7. At this stage, it does appear that PLC is absolutely required.

Besides DAG and PLC, TRPC3/6/7 are also under regulation of many other factors, including IP3R, Ca2+/CaM, cGMP/PKG, phosphorylation and glycosylation status, and etc. Similar to other TRPCs, CIRB site is also present in the C-termini of TRPC3/6/7 (Tang et al., 2001). Functional studies with TRPC3 have shown that in human embryonic kidney 293 (HEK293) cells stably expressing TRPC3, IP3R competed with inhibitory CaM to bind to the CIRB site, and the binding of IP3R to TRPC3 activated the channel (Zhang et al., 2001). Contradictorily, in DT40 cells lacking all three types of IP3Rs, TRPC3 could still function normally (Wedel et al., 2003), arguing against the essential role of IP3R for TRPC3 activation.

Ca2+ regulates TRPC3/6/7 from both extracellular and intracellular sides in a complex manner. To successfully record TRPC3 current, a narrow range of intracellular [Ca2+] concentrations from 100 nM to 400 nM was required for optimal results. On the other hand, the activation of TRPC6 and TRPC7 was potentiated by an increase of extracellular [Ca2+] from 0.1 to 1 mM and
intracellular \([\text{Ca}^{2+}]\) from 20 to 200 nM, but a further increase of extracellular or intracellular \([\text{Ca}^{2+}]\) inhibited channel activities (Shi et al., 2004). The underlying mechanism for this complex regulation by \(\text{Ca}^{2+}\) may involve \(\text{CaM}\), but further investigations are required.

When heterologously expressed in HEK293 cells, TRPC3 showed a high constitutive activity, whereas TRPC6 was tightly receptor regulated (Dietrich et al., 2003). The underlying mechanism for this difference is due to the distinct N-linked glycosylation patterns between TRPC3 and TRPC6. Two consensus glycosylation sites (NX(S/T)) are present in TRPC6: Asn473 located in the first extracellular loop corresponding to the single glycosylation site Asn418 in TRPC3, and Asn561 in the second extracellular loop, which is not present in TRPC3 (Dietrich et al., 2003; Vannier et al., 1998). A point mutation of the Asn561 to Gln in TRPC6 converted the tight controlled TRPC6 to highly constitutively active (Dietrich et al., 2003). Therefore, the basal activities of TRPC3/6/7 are regulated by protein glycosylation.

TRPC3/6/7 are also regulated by phosphorylation. It has been reported that when TRPC3 was co-expressed with protein kinase G (PKG) in HEK293 cell, cGMP inhibited TRPC3 by activating PKG and phosphorylating two consensus PKG phosphorylation sites: Thr11 and Ser263 (Kwan et al., 2004). For TRPC6, phosphorylation of Ser768 greatly inhibited carbacol (CCh)-induced TRPC6 activity (Estacion et al., 2004; Kim & Saffen, 2005). On the other hand,
phosphorylation could also facilitate the activities of TRPC3/6/7. Fyn, a Src family protein tyrosine kinase, physically interacted with TRPC6 and increased channel activities through phosphorylation (Hisatsune et al., 2004).

1.4.1.6 The activation and regulation of TRPC4, C5

In contrast to TRPC3/C6/C7, TRPC4 and TRPC5 are inhibited by DAG (Venkatachalam et al., 2003; Zhu et al., 2005). Uniquely, TRPC4 and TRPC5 activities are greatly potentiated by lanthanides at micromolar range, which typically block almost all other TRP channels (Jung et al., 2003). Based on current knowledge, there are 3 major activation modes for TRPC4 and TRPC5: receptor-mediated mode, store-operated mode, and agonist-evoked mode.

1.4.1.6.1 Receptor-mediated activation

Similar to other TRPC channels, multiple lines of evidence have shown that when TRPC4/5 was co-expressed with G_{q/11}-coupled receptors such as histamine receptor type 1, H_{1}R (Obukhov & Nowycky, 2004), muscarinic receptor type 3, M_{3}R (Lee et al., 2003), P_{2}Y purinergic receptors (Okada et al., 1998), and bradykinin receptor type 2, B_{2}R (Ohta T et al., 2004), application of the corresponding agonists activated TRPC4/5 currents. Moreover, intracellular infusion of GTP\gamma S, a non-metabolizable analog of GTP that maintains Gα subunits in the active GTP-bound forms, is commonly used to activate TRPC4 and TRPC5 (Zhu et al., 2005; Obukhov & Nowycky, 2004; Lee et al., 2003; Schaefer et al., 2000), indicating that active G proteins are sufficient to activate
TRPC4 and TRPC5. In addition to G<sub>q/11</sub>-coupled receptors, receptor tyrosine kinases, such as epithelial growth factor (EGF) receptor, were also shown to mediate the translocation and activation of TRPC5 (Bezzerides et al., 2004). The common signaling molecule involved in both G<sub>q/11</sub>-coupled receptor and receptor tyrosine kinase signaling pathway is PLC, with different PLC isoforms being recruited. A PLC inhibitor, U73122, blocked the activity of TRPC4/5 induced by both G<sub>q/11</sub>-coupled receptor pathway and receptor tyrosine kinase pathway (Schaefer et al., 2000), again, supporting the essential role of PLC in the activation of TRPC subfamily channels.

1.4.1.6.2 Store-operated activation

A number of experimental results also support that TRPC4 and TRPC5 function as SOCs. In TRPC4 knockout mice, it was found that a native endothelial SOC was abolished (Freichel et al., 2001); moreover, introduction of TRPC4 antisense DNA into bovine adrenal gland where TRPC4 was highly expressed greatly reduced endogenous SOC (Philipp et al., 2000). However, there are also many reports showing that TRPC4 and TRPC5 are not sensitive to store-depletion and they do not function as SOCs. For instance, Strubing et al. (2001) reported that store depletion induced by either IP<sub>3</sub> or thapsigargin application had no effect on TRPC5 activation. The ineffectiveness of IP<sub>3</sub> and thapsigargin for TRPC5 activation was reproduced by another group (Venkatachalam et al., 2003).
1.4.1.6.3 Agonist-evoked activation

The third mode of activation of TRPC4/5 is achieved through direct agonist effect. Four major types of agonists have been reported: lanthanides, lysophosphatidylcholine, some nitrosylation reagents, and an endogenous redox protein thioredoxin.

Lanthanum or gadolinium in 1-100 micromolar range greatly potentiated TRPC4 and TRPC5 currents, but higher concentrations of lanthanides inhibited TRPC4/C5 (Jung et al., 2003). By systematically screening all negatively charged amino acids located in the extracellular loops of TRPC5, Jung et al. have identified three residues responsible for the facilitatory effects of lanthanides: Glu543, Glu595, and Glu598. Importantly, these residues were located close to the outer mouth of the channel pore and they are conserved in TRPC4 as well (Jung et al., 2003). It was proposed that these negatively charged glutamate residues were also sensitive to other extracellular cations such as Ca\(^{2+}\) and Mg\(^{2+}\) under physiological conditions (Jung et al., 2003), thus providing a regulation mechanism for TRPC4/5 by extracellular cations.

TRPC5 was also shown to be directly activated by some endogenous lysophospholipids, such as lysophosphatidylcholine, independent of G protein-coupled receptors (Flemming et al., 2006). However, the precise mechanism for TRPC5 activation by lysophospholipids is still elusive.
The third type of direct activator for TRPC4 and TRPC5 was discovered by Mori’s group (Yoshida et al., 2006). Nitric oxide (NO) was found to activate TRPC5 by cysteine S-nitrosylation at two conserved sites, Cys553 and Cys558 (Yoshida et al., 2006). When perfused extracellularly, several membrane permeable reactive disulfides, including 2,2'-dithiobis(5-nitropyridine) (5-nitro-2-PDS), 2,2'-dithiodipyridine (2-PDS), and 4,4'-dithiodipyridine (4-PDS), were shown to nitrosylate these two cysteines and mimic the effects of endogenous NO on the activation of TRPC5. In contrast, membrane impermeable analog 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) had no effect on the activation of TRPC5 (Yoshida et al., 2006), supporting the intracellular localization of Cys553 and Cys558.

Very recently, another endogenous agonist for TRPC5 was reported by Beech’s group (Xu et al., 2008). These investigators also studied the conserved Cys553 and Cys558 in TRPC5 discovered by Mori’s group. Since these two cysteines are very close, they reasoned that a disulfide-bond might form under resting conditions. They found that extracellular perfusion of a membrane-impermeable disulphide reducing agent Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) was able to directly activate TRPC5, supporting the extracellular localization of Cys553 and Cys558, which contradicts Mori’s conclusion. By mutating either cystein residue which abolished the disulfide-bond formation, TRPC5 became constitutively active and not sensitive to any reducing reagents they used, suggesting that TRPC5 may function as a redox sensor by forming and breaking disulfide-bond between these two conserved cysteines. Xu et al. further identified
an endogenous reducing protein as the direct activator of TRPC5-thioredoxin, which was secreted and present in the synovial fluid from patients with arthritis. Thioredoxin was shown to directly activate endogenous TRPC5-like current in fibroblast-like synoviocytes (FLS cells) by breaking the disulfide-bond (Xu et al., 2008). Since discrepancy exists regarding the location of these two cysteines, more studies are required to understand the precise roles of these residues.

1.4.1.6.4 Regulatory factors of TRPC4/5

TRPC4 and TRPC5 have multiple binding partners, including CaM (Tang et al., 2001), IP₃Rs (Tang et al., 2001), NHERF (Tang et al., 2000), enkurin (Sutton et al., 2004), junctate (Stamboulian et al. 2005), immunophilin FKBP52 (Sinkins et al. 2004), and another TRPC subtype-TRPC1.

Most Ca²⁺ permeable channels are under regulation by Ca²⁺ through CaM, and there is no exception for TRPC4/5. Ca²⁺ could be facilitatory for TRPC4/5. For example, an increase of intracellular [Ca²⁺] from 10 nM to 200 nM significantly increased TRPC5 activity (Strubing et al., 2001). Ionomycin, a Ca²⁺ ionophore which induces Ca²⁺ release from ER store, directly activated TRPC5 expressed in *Xenopus* oocyte (Kinoshita-Kawada et al., 2005). However, a further increase of intracellular [Ca²⁺] from 400 nM to 5 μM significantly inhibited TRPC5 current (Ordaz et al., 2005). Thus, depending on the local Ca²⁺ concentration, intracellular Ca²⁺ plays a dual role on TRPC5 activation—either facilitatory or inhibitory. Moreover, the activation of TRPC4/5 by other stimuli requires a
minimum intracellular [Ca\textsuperscript{2+}]: if intracellular [Ca\textsuperscript{2+}] was buffered at 10 nM, TRPC4 and TRPC5 are not responsive to any other stimuli (R. Xiao, personal observation). Therefore, it seems that intracellular Ca\textsuperscript{2+} plays a permissive role for the activation of TRPC4/5. The mediator for intracellular Ca\textsuperscript{2+} to regulate TRPC4/5 is most likely CaM. Three, four, and two CaM-binding sites have been identified for TRPC5, TRPC4\textalpha, and TRPC4\textbeta, respectively (Zhu, 2005). Although little is known for the precise function of each CaM-binding site, it is possible that different CaM-binding sites have distinct, or even opposite, effects.

Another important endogenous regulator for TRPC4/C5 is TRPC1. TRPC1 forms hetero-tetramers with both TRPC4 and TRPC5 \textit{in vitro} and \textit{in vivo}. The formation of heteromers between TRPC1 and TRPC4/5 greatly changes the current-voltage (I-V) relationship of TRPC4/C5: the I-V curves of TRPC4 and TRPC5 are double-rectifying, while the I-V curves for TRPC1-TRPC4 and TRPC1-TRPC5 heteromers are strongly outwardly-rectifying (Strubing et al., 2001). Considering the co-localization of TRPC1 and TRPC4/5 channels in many tissues, the dynamic changes of TRPC1 and TRPC4/5 channel compositions might have important physiological implications.

TRPC4 and TRPC5 are activated following GPCR activation, but a continuous perfusion of the agonist to G protein-coupled receptor typically leads to inactivation (or desensitization) of TRPC4/5. The underlying mechanism for this inactivation involves DAG/PKC pathway (Zhu et al., 2005). By perfusing
TRPC4/5 expressing cells with membrane permeable DAG analog OAG or increasing endogenous DAG level by inhibiting DAG kinase, TRPC4 and TRPC5 activity was completely abolished (Venkatachalam et al., 2003), indicating an inhibitory effect of DAG for TRPC4/5. This inhibitory effect of DAG for TRPC4/5 occurred through PKC and the phosphorylation at Thr792 of TRPC5 by PKC might underlie the inactivation process (Zhu et al., 2005).

1.4.2 Activation and regulation of TRPV channels
1.4.2.1 Activation and regulation of TRPV1
At least five modes of activation exist for TRPV1, making TRPV1 the best example of polymodal sensors. These activation modes include: vanilloids from natural plants, high temperature, G protein-coupled receptors, voltage, some endogenous lipids, and protons.

1.4.2.1.1 Activation of TRPV1 by natural plant ingredients
A number of vanilloids from natural plants have been identified to specifically activate TRPV1 among TRPV subfamily, including capsaicin from chili pepper and resiniferatoxin from resin spurge (*Euphorbia resinifera*). Besides, olvanil, a capsaicin analog, also strongly activates TRPV1. These vanilloids are all hydrophobic molecules and they activate TRPV1 by binding to several specific amino acids. Chicken TRPV1 (cTRPV1) is not sensitive to capsaicin, although it maintains all other functional properties of rodent TRPV1 (Jordt & Julius, 2002). By comparing the amino acid sequences of cTRPV1 and rat TRPV1, Julius and
his colleague have identified three essential residues for capsaicin sensitivity: Arg491 in transmembrane segment 2 (S2), Tyr511 and Ser512 in the transition between the second intracellular loop and S3. Point mutations of these three residues abolished the sensitivity of rat TRPV1 to capsaicin and the binding of rat TRPV1 to radio-labeled super-potent vanilloid agonist-RTX (Jordt & Julius, 2002).

Besides highly specific vanilloid agonists, such as capsaicin and RTX, some other compounds from plants also activate TRPV1 in a less-specific and less-potent manner. These compounds include camphor from the wood of the camphor laurel (Cinnamomum camphora), which also activates TRPV3 (Xu et al., 2005), allicin from raw garlic, which also activates TRPA1 (Macpherson et al., 2005). The mechanism for these non-specific compounds to activate TRPV1 is unknown.

1.4.2.1.2 Activation of TRPV1 by heat

Theoretically, all molecules are sensitive to heat due to the thermodynamic effects. To define a real temperature-gated ion channel, researchers have used Q10 (temperature coefficient over a 10°C range) values to evaluate the heat sensitivity. To claim a channel as being heat-gated, the Q10 value should be larger than two; otherwise, it is simply due to regular thermodynamic effect (Hille, 2001). Endogenous TRPV1 in rat DRG neurons has an activation temperature threshold of 43°C (Caterina et al., 1997), and the Q10 value for rat TRPV1 was determined to be 25.6 (Vlachová et al., 2003).
Is there any specific motif within the TRPV1 molecule to underlie the heat sensitivity of the channel? Or is a global conformational change responsible for the heat sensitivity? Many researchers in the field are interested in molecular mechanisms responsible for the heat sensing property of TRPV1, but so far little firmly established evidence is available. It was shown that the C-terminus of TRPV1 might be responsible for the thermosensation of the channel: by swapping the C-termini between heat-activated TRPV1 and cold-activated TRPM8, Brauchi et al. found that a chimera of TRPV1 that contained the TRPM8 C-terminus became cold-activated, while a chimera of TRPM8 that contained the TRPV1 C-terminus became heat-activated (Brauchi et al., 2006). However, no follow-up experiments from other laboratories have confirmed this important observation.

1.4.2.1.3 Activation of TRPV1 by voltage

TRP channels were initially described as a voltage-insensitive Ca^{2+} influx pathway (Wes et al., 1995; Zhu et al., 1995). Indeed, most TRP channels are not voltage-gated. However, TRPV1 displayed some interesting voltage-dependent properties (Voets et al., 2004). For example, TRPV1 was activated by high positive potentials without any other stimulus (Voets et al., 2004). More interestingly, other chemical or physical stimuli work in a synergistic manner with voltage on activating TRPV1. For example, heat or capsaicin treatment greatly reduced the depolarizing voltage threshold required for TRPV1 activation (the
voltage for half-maximal activation, $V_{1/2}$, was changed from +150 mV to +10.6 mV with the addition of 50 nM capsaicin) (Voets et al., 2004). This important finding indicates that TRPV1 might function as a voltage-gated Ca$^{2+}$ permeable channel in the presence of other stimuli in vivo: for example, during tissue injury, multiple inflammatory factors, including histamine, proton, ATP, and bradykinin, are released and function as stimulators of TRPV1. Under these conditions, TRPV1 can be activated by a slight depolarizing force and provides a further cation influx and larger depolarization.

Strikingly, TRPM8, a cold-activated TRP channel, shows similar voltage-dependent properties as TRPV1 (Voets et al., 2004). TRPM8 is also activated by highly positive potential in the absence of other stimuli. However, cold or menthol treatment greatly shifted the voltage activation threshold of TRPM8 to more negative (for instance, the $V_{1/2}$ for TRPM8 was changed from +200 mV at 37°C to +25 mV at 8°C ) (Voets et al., 2004). These results led to a generalized proposal that the heat sensitivity of thermo-TRP channels is tightly coupled to the voltage-dependent gating. The activation of thermo-TRPs by other physical or chemical stimuli was suggested to occur through a left shift of conductance-voltage (G-V) relationship of these TRP channels (Voets et al., 2004; Nilius et al., 2005). In this theory, voltage is the final determinant for the activation of thermo-TRPs. However, it is always somewhat risky to try to generalize because exceptions always exist. Recently, another group revealed voltage-independent activation of TRPV1 and TRPM8 at very high agonist concentrations. They proposed that
voltage, similar to other stimuli, is a partial activator of thermo-TRPs and there is no final determinant for the activation of thermo-TRPs. Distinct sources of stimuli activate thermo-TRPs in a concerted manner (Matta & Ahern, 2007).

1.4.2.1.4 Activation of TRPV1 by lipids

Several endogenous fatty acids with structural homology with capsaicin have been identified to activate TRPV1, including endocannabinoid anandamide (Zygmunt et al., 1999), N-arachidonoyl dopamine (NADA) (Price et al., 2004), oleoyldopamine (Szolcsányi et al., 2004), oleoylethanolamide (OEA) (Movahed et al., 2005), and 12-hydroperoxyeicosatetraenoic acid (12-HPETE) (Sexton et al., 2007). These fatty acids may function as the endogenous agonists for TRPV1 with a similar activation mechanism to that of capsaicin.

1.4.2.1.5 Activation of TRPV1 by protons

At low concentrations between pH6-7, protons greatly potentiated TRPV1, whereas at higher concentrations (pH<6), TRPV1 was directly activated by protons (Jordt et al., 2000). Two critical negatively charged extracellular glutamate residues are responsible for the proton sensitivity of TRPV1. Glu600 at the region linking S5 and the putative pore-forming loop appears to underlie the potentiation effect of protons, while Glu648 at the putative pore loop seems essential for the direct activation of TRPV1 by protons (Jordt et al., 2000). Importantly, protons shifted the temperature threshold of TRPV1 activation from 43°C to 30-32°C (Jordt et al., 2000), showing that TRPV1 can be activated at
normal body temperature (37°C) under acidification condition normally caused by tissue injury.

1.4.2.1.6 Regulatory mechanisms of TRPV1

The activation of multiple type of receptors coupled to PLC signaling pathway, such as Gq/11-coupled bradykinin receptor B₂R, histamine receptor H₁R, purinergic receptor P₂Y₁, and receptor tyrosine kinase-coupled nerve growth factor (NGF) receptor TrkA/p75, are all shown to potentiate TRPV1 (Chuang et al., 2001). The molecular mechanism underlying this potentiation appears to involve PLC-induced PIP₂ hydrolysis, which releases the inhibition of TRPV1 by PIP₂ (Chuang et al., 2001). The inhibition of TRPV1 by PIP₂ is due to the direct binding of PIP₂ to amino acids 778-819 at the C-terminus of TRPV1 (Prescott & Julius, 2003). Mutations in this region that weakened the interaction between PIP₂ and TRPV1 greatly potentiated the activation of TRPV1 by both chemical and heat stimuli (Prescott & Julius, 2003). However, the inhibitory effect of PIP₂ to TRPV1 was disputed recently by several other groups. In contrast, a stimulatory effect of PIP₂ to TRPV1 was repeatedly reported (Stein et al., 2006; Lukacs et al., 2007). It turned out that PIP₂ had dual roles for the activation of TRPV1: being inhibitory at a low level of stimulation and facilitatory at the high level of stimulation (Lukacs et al., 2007).

TRPV1 is also under extensive regulation by phosphorylation/dephosphorylation. Multiple types of kinases have been found to regulate TRPV1, including
Ca²⁺/CaM-dependent kinase II (CaMKII) (Jung et al., 2004), PKA (Vetter et al., 2006), PKC (Mandadi et al., 2006), mitogen activated protein (MAP) kinase (Zhu & Oxford, 2007), Src-family kinases (Katsura et al., 2006), and cyclin-dependent kinase 5 (Cdk5) (Pareek et al., 2007). These kinases phosphorylate TRPV1 at distinct sites, but interestingly, all phosphorylation events by distinct kinases lead to the potentiation of channel function. In contrast, dephosphorylation by calcineurin (a phosphatase) has been reported to mediate the Ca²⁺-dependent desensitization of TRPV1 (Mohapatra & Nau, 2005).

1.4.2.2 Activation and regulation of TRPV2
To date, relatively little is known regarding the activation and regulation of TRPV2. Partly, this is due to the difficulty to express TRPV2 in mammalian cells and most functional data were obtained from *Xenopus* oocyte. TRPV2 was shown to be activated by high temperatures with the activation threshold of 52ºC, which is unlikely to happen under physiological conditions (Caterina et al., 1999). In contrast to TRPV1, TRPV2 was not sensitive to capsaicin or protons (Caterina et al., 1999). No specific chemical agonist has been found for TRPV2 so far. The only available non-specific agonist for TRPV2 is 2-aminoethoxydiphenyl borate (2-APB) (EC₅₀ value is about 130 μM) (Hu et al., 2004).

1.4.2.3 Activation and regulation of TRPV3
As a thermo-sensitive TRP channel, TRPV3 is also activated by heat, with an activation threshold of about 32ºC (Peier et al., 2002b; Xu et al., 2002; Smith et
al., 2002). Normal body temperature is higher than the activation threshold of TRPV3, indicating that TRPV3 is either constantly active or some inhibitory factors are present to regulate the channel. A unique feature of TRPV3 is that the activation of TRPV3 is strongly sensitized upon repetitive stimulation (Peier et al., 2002b; Xu et al., 2002), and the underlying mechanism is one of the major studies in this thesis.

Besides innocuous heat, some chemical compounds have been identified to activate TRPV3. For example, several vanilloids such as camphor, oregano, thyme and clove-derived flavors and skin sensitizers were shown to activate TRPV3 in a non-specific and somewhat ineffective way (Moqrich et al., 2005; Xu et al., 2006). Several synthetic chemicals activate TRPV3 more efficiently. Two independent groups reported that 2-APB, commonly used as a non-specific IP$_3$R blocker, activated TRPV3 both in vitro and in vivo with an EC$_{50}$ value of about 40 μM (Chung et al., 2004; Hu et al, 2004). DPBA, a 2-APB analog, was also shown to activate TRPV3 with a slightly higher efficiency (Chung et al., 2005). The action site(s) for all TRPV3 chemical agonists still remains elusive.

The regulation of TRPV3 is one of the major topics in my graduate study, and it will be discussed in details.

1.4.2.4 Activation and regulation of TRPV4
Similar to TRPV3, TRPV4 is activated by innocuous warm temperature, with a temperature threshold of activation at about 28°C (Watanabe et al., 2002). TRPV4 is also activated by multiple other factors, such as cell swelling (Liedtke W et al., 2000), 4-phorbol 12,13-didecanoate (4α-PDD) (Watanabe et al., 2002), endocannabinoid anandamide (Watanabe et al., 2003), arachidonic acid metabolites (Watanabe et al., 2003), and bisandrogropholide from a traditional Chinese medicine Andrographis (Smith et al., 2006).

Nilius and colleagues studied the molecular mechanisms of the activation of TRPV4 by swelling, heat, and chemical agonists. They found that different stimuli recruited distinct signaling pathways for the activation of TRPV4 (Vriens et al., 2004). Swelling activated TRPV4 by using a phospholipase A2 (PLA2) and cytochrome p450 epoxygenase-dependent pathway, whereas chemical agonist 4α-PDD activated TRPV4 by directly binding to tranmembrane domain S3 in a similar fashion as capsaicin activating TRPV1 (Vriens et al., 2004).

Similar to several other TRP channels, TRPV4 is under complex regulation of Ca\(^{2+}\) from both extracellular and intracellular sides. The activation of TRPV4 required the presence of extracellular Ca\(^{2+}\) (Strotmann et al., 2000; Strotmann et al., 2003). However, an elevation of intracellular [Ca\(^{2+}\)] inhibited TRPV4 current (Watanabe et al., 2003). The detailed mechanism for TRPV4 to sense extracellular and intracellular Ca\(^{2+}\) concentration changes remains unknown.
1.4.2.5 Activation and regulation of TRPV5 and TRPV6

In contrast to TRPV1-4, TRPV5 and TRPV6 are not directly activated by heat and they display a high Ca$^{2+}$ selectivity over Na$^+$. Currently, there is no specific agonist or antagonist available for either TRPV5 or TRPV6. Functionally, TRPV5 and TRPV6 are important for Ca$^{2+}$ re-absorption in kidney and small intestine, and their expression levels are controlled by several hormones such as vitamin D3, estrogen, thyroid hormone, and parathyroid hormone (PTH) (Hoenderop et al., 2005). For instance, vitamin D3 was shown to up-regulate TRPV5 expression in kidney (Hoenderop et al., 2001).

Both TRPV5 and TRPV6 display voltage dependence due to the reversible intracellular and extracellular Mg$^{2+}$ block/unblock (Voets et al., 2003; Pedersen et al., 2005). Extracellular Mg$^{2+}$ binds to a negatively charged residue Asp542 in the putative selectivity filter region of TRPV5 and blocks the channel. After mutating Asp542 to Ala, the mutant channel lost Ca$^{2+}$ permeability and extracellular Mg$^{2+}$ block (Nilius et al., 2001). Intracellular Mg$^{2+}$ also blocks TRPV6, and this block is relieved under positive potentials because Mg$^{2+}$ is able to pass through the selectivity filter with the outward driving force (Voets et al., 2003).

Ca$^{2+}$-dependent inactivation is a common feature for TRPV5 and TRPV6. The underlying mechanism could be due to a direct binding of Ca$^{2+}$/CaM to the channels. CaM has been shown to bind to both TRPV5 and TRPV6 in a Ca$^{2+}$-dependent manner (Lambers et al., 2004).
1.4.3 Activation and regulation of TRPM channels

1.4.3.1 Activation and regulation of TRPM1 and TRPM3

No established functional study has been reported on the activation or regulation of TRPM1. For TRPM3, D-erythro-sphingosine (SPH) is the only established useful agonist so far (Grimm et al., 2005). Intracellular Mg\textsuperscript{2+} seems to block the constitutive activity of TRPM3 (Oberwinkler et al. 2005), but the site of action for intracellular Mg\textsuperscript{2+} is unknown.

1.4.3.2 Activation and regulation of TRPM2 and TRPM8

In the TRPM subfamily, TRPM2 and TRPM8 are most intensively studied. Many activation and regulation mechanisms for TRPM2 and TRPM8 have been proposed and validated. In general, similar to TRPV1, TRPM2 and TRPM8 are both polymodal sensors-multiple independent activation modes are present for them.

TRPM2 has been shown to be directly activated by ADP-ribose (ADPR), H\textsubscript{2}O\textsubscript{2} and oxidative stress. TRPM2 has an intrinsic ADPR pyrophosphatase domain within its C-terminus, making TRPM2 both an ion channel and an enzyme (Perraud et al., 2001). ADPR binds to the enzymatic domain of TRPM2 and activates the channel both in vitro and in vivo (Perraud et al., 2001). H\textsubscript{2}O\textsubscript{2} and other oxidative stress also activate TRPM2 (Hara et al., 2002). However, the molecular mechanism of action for H\textsubscript{2}O\textsubscript{2} and oxidative stress is still in debate: it
could be due to the indirect effect of H$_2$O$_2$-induced ADPR generation or some unknown direct effect of H$_2$O$_2$ on TRPM2. Intracellular Ca$^{2+}$ did not activate TRPM2 by itself, but the presence of relatively high [Ca$^{2+}$] due to receptor activation greatly potentiated the activation effect of ADPR and shifted the EC$_{50}$ of ADPR from 50 μM to 90 nM (Kolisek et al., 2005). However, the mechanism for TRPM2 to detect intracellular Ca$^{2+}$ is unclear.

TRPM8, opposite to TRPV1, is activated by cold temperature and the cooling compound menthol from natural plant mint. The activation modes of TRPM8 include cold activation, direct chemical agonist activation, and voltage activation.

TRPM8 is the primary environmental cold sensor and has a temperature threshold of activation at about 25ºC (McKemy et al. 2002; Bautista et al., 2007). However, the temperature threshold of TRPM8 activation is significantly changed depending on the transmembrane voltage: for example, the temperature threshold is much higher at positive potentials than that at negative potentials (Voets et al., 2004). Voets et al have proposed that the effect of cooling temperature on the activation of TRPM8 is through a gradual left-shift of the V$_{1/2}$ (for instance, cooling from 37ºC to 15.8ºC leads to a decrease of 150 mV in V$_{1/2}$), and voltage sensitivity is the governing mechanism responsible for the cold sensitivity of TPRM8 (Voets et al., 2004).
Cooling compound menthol and its derivatives are known to activate the cold receptor TRPM8 which explains the cooling sensations caused by these compounds. Menthol activates TRPM8 with an EC\textsubscript{50} value at micromolar range (McKemy et al., 2002). The important residues of TRPM8 for menthol action have been determined: Tyr745 in S2, Tyr1005 and Leu1009 in the TRP domain (Bandell et al., 2006). Using systematic random mutagenesis, Bandell et al. have found that point mutations of above three residues specifically abolished the menthol sensitivity of TRPM8 without affecting the normal cold response. They proposed that similar to the binding of capsaicin to Arg491 in S2 of TRPV1, Try745 might be involved in the direct binding of menthol to TRPM8. Tyr1005 and Leu1009 might be downstream of menthol binding and involved in the regulation of channel pore (Bandell et al., 2006). Icilin, a synthetic super-cooling compound, activates mammalian TRPM8 with an EC\textsubscript{50} value at nanomolar range (McKemy et al., 2002). Unlike rodent TRPM8, chicken TRPM8 is not sensitive to icilin although it still maintains the sensitivity to cold and menthol (Chuang et al., 2004). By comparing the amino acid sequences between rat TRPM8 and chicken TRPM8, Chuang et al. identified the icilin-binding site in rat TRPM8 (Gly805 in S3), and the counterpart residue in chicken TRPM8 was Ala796 (Chuang et al., 2004). After replacing Gly805 with an alanine in rat TRPM8, icilin sensitivity of rat TRPM8 was completely abolished while menthol and cold sensitivities remained normal; reversely, substitution of Ala796 with a glycine in chicken TRPM8 made the channel icilin sensitive (Chuang et al., 2004).
TRPM8 is regulated by multiple intracellular factors, including Ca$^{2+}$, protons, PIP$_2$, and kinases. Intracellular Ca$^{2+}$ is particularly important for icilin-induced TRPM8 activation. It was found that the activation of TRPM8 by icilin required a concomitant elevation of intracellular [Ca$^{2+}$], whereas menthol did not require intracellular Ca$^{2+}$ for its action (Chuang et al., 2004). Although it is possible that intracellular Ca$^{2+}$ regulates TRPM8 through CaM, no established study has been reported to demonstrate it so far. Like many other regulatory factors, PIP$_2$ has an opposite effect on TRPM8 compared with TRPV1. PIP$_2$ application directly activated TRPM8 while the hydrolysis of PIP$_2$ via receptor activation inhibited TRPM8 (Liu & Qin, 2005). PIP$_2$ was shown to directly bind to the TRP domain of TRPM8. Strikingly, the conserved TRP domain of TRPM5 and TRPV5 were also shown to bind to PIP$_2$, which led to a hypothesis that the function of TRP domains is related to the PIP$_2$ sensitivity (Rohács et al., 2005). However, this view was disputed later (Nilius et al., 2006). Opposite to the sensitization effect on TRPV1, phosphorylation of TRPM8 by PKC, PKA, and other kinases has been shown to be responsible for the desensitization of the channel (Premkuma et al., 2005; Abe et al., 2006; Mizushima et al., 2006). Protons also regulate TRPM8: the activation of TRPM8 by icilin and cold but not menthol was inhibited by a change of intracellular pH from 7.3 to 6.5 (Andersson et al., 2004).

1.4.3.3 Activation and regulation of TRPM4 and TRPM5

The first known direct activator for TRPM4 and TRPM5 is intracellular Ca$^{2+}$. The activation of G$_{q/11}$-coupled receptors was repeatedly shown to induce TRPM4
and TRPM5 current both in heterologously expressed cells and native cells (Liu & Liman, 2003; Zhang et al., 2003). PLCβ is activated downstream of the activation of Gq/11-coupled receptors. PIP2 is hydrolyzed by PLCβ to generate DAG and IP3. DAG activates PKC and IP3 releases Ca2+ from ER Ca2+ store through binding to IP3Rs. Among all above signaling molecules downstream of Gq/11-coupled receptors, only Ca2+ was shown to directly activate TRPM4 and TRPM5 (Liu & Liman, 2003; Launay et al., 2002). The most convincing evidence was that TRPM4 and TRPM5 were activated by Ca2+ in cell-free inside-out patches where all cellular contents were supposed to be lost (Liu & Liman, 2003; Nilius et al., 2003). However, the action site(s) of intracellular Ca2+ on TRPM4 and TRPM5 is still elusive.

Interestingly, TRPM4 and TRPM5 were also shown to be activated by warm temperature (Talavera et al., 2005). Similar to other heat-activated TRP channels, V1/2 of TRPM4 and TRPM5 is largely left-shifted with the increase of temperature: for instance, the V1/2 of TRPM5 decreased from +250 mV to +80 mV when temperature was increased from 15ºC to 35 ºC (Talavera et al., 2005).

TRPM4 and TRPM5 are also regulated by PIP2. A consistent observation from electrophysiological recordings of TRPM4 and TRPM5 was the Ca2+-dependent run-down following channel activation (Liu & Liman, 2003; Hofmann et al., 2003; Nilius et al., 2004). PIP2 appears to be responsible for this desensitization process. In inside-out patches, direct application of PIP2 significantly increased
Ca\textsuperscript{2+} sensitivity and current size of TRPM5 during run-down, but it had no effect before desensitization happened for the channel (Liu & Liman, 2003). Similar results for TRPM4 were reported by two independent groups (Zhang et al., 2005; Nilius et al., 2005). Given that PIP\textsubscript{2} also regulates other TRP channels including TRPV1, TRPM8, and TRPM7, it has been proposed that PIP\textsubscript{2} might be a common regulator of all TRP channels (Hardie, 2003).

1.4.3.4 Activation and regulation of TRPM6 and TRPM7

TRPM6 and TRPM7 are important for Mg\textsuperscript{2+} homeostasis (Schlingmann et al., 2007). Knockout of endogenous TRPM7 in DT40 cells caused cell death which indicated its essential role in cell survival (Nadler et al., 2001; Schmitz et al., 2003). Both TRPM6 and TRPM7 are gated by intracellular Mg\textsuperscript{2+} and Mg-complexed nucleotide in an inhibitory manner: TRPM6 and TRPM7 have a low level of constitutive activity under resting condition, however, by reducing the concentration of intracellular Mg\textsuperscript{2+} or Mg-ATP nucleotide, large TRPM6 and TRPM7 currents are induced (Nadler et al., 2001). The inhibitory effect of Mg-ATP is not due to the hydrolysis of ATP because a non-metabolizable analog Mg-ATP\textsubscript{γ} also blocks TRPM7 (Nadler et al., 2001). Interestingly, Na-ATP was shown to activate TRPM7 and it was proposed that the channel was activated by ATP-dependent phosphorylation (Runnels et al., 2001). However, this proposal was disputed because no other established study has confirmed the essential role of phosphorylation for the activation of TRPM7. In contrast, ATP is a chelator of Mg\textsuperscript{2+}. The stimulatory effect of Na-ATP is likely due to the reduction of
intracellular [Mg^{2+}] by chelating Mg^{2+} ions and releasing the channel from the inhibition of Mg^{2+} block. Indeed, two other Mg^{2+} chelators, EGTA and HEDTA, did the same as Na-ATP and induced a maximal activation of TRPM7 (Demeuse et al., 2006). An Mg-ATP-binding site of TRPM7 has been identified in the kinase domain of the channel. Two point mutations within this domain K1648R and G1799D greatly reduced the inhibitory effects by Mg-ATP (Demeuse et al., 2006).

Both TRPM6 and TRPM7 have an intrinsic kinase domain in their C-termini and this kinase domain can auto-phosphorylate the channels (Nadler et al., 2001; Schlingmann & Gudermann, 2005). The functional significance of this intrinsic kinase domain is controversial: Runnels et al. showed that the kinase domain was required for the channel function because mutations designed to abolish the phosphotransferase activity in this domain caused the channel to be non-functional (Runnels et al., 2001); however, two other studies reported that TRPM7 mutants without phosphotransferase activity were still functional as the wild type channel (Schmitz et al. 2003; Matsushita et al. 2005).

PIP2 was also shown to be important to maintain TRPM7 channel function. Constitutive TRPM7 current was inhibited by PIP2 hydrolysis caused by the activation of G_{q/11}-coupled receptors (Runnels et al., 2002).

1.4.4 Activation and regulation of TRPA1 channel
Similar to TRPV1 and TRPM8, multiple activation modes exist for TRPA1, including direct chemical agonist activation, noxious cold temperature activation, G protein-coupled receptor activation, and mechanic force activation.

1.4.4.1 Activation by cysteine modification

TRPA1 are sensitive to multiple naturally occurring and synthetic compounds and most of these compounds are considered pungent. Allyl-isothiocyanate, the pungent ingredient from mustard oil, specifically activated TRPA1 with an EC$_{50}$ of about 10 $\mu$M (Jordt et al., 2004). Allicin, the pungent ingredient from fresh but not baked garlic, activated TRPA1 with an EC$_{50}$ of about 1 $\mu$M (Macpherson et al., 2005). Acrolein, an environmental irritant present in tear gas and vehicle exhaust, also activated TRPA1 with an EC$_{50}$ of about 5 $\mu$M (Bautista et al., 2006). Notably, the above three chemicals, together with many other TRPA1 agonists, are not necessarily structural related.

A question arises: how can a molecule sense so many different stimuli from distinct sources? Part of the reason might be due to the covalent modification of some cysteine residues of TRPA1 by some of these compounds. Two independent groups have found that most TRPA1 agonists were membrane permeable and able to covalently bind to three intracellular cysteines: Cys415, Cys422 and Cys622 of mouse TRPA1 (Macpherson et al., 2007), and Cys619, Cys639, and Cys663 of human TRPA1 (Hinman et al., 2006). Although there is some discrepancy on the precise sites of the cysteines, both groups have
mapped the binding sites of chemical reactive agonists to be within the N-terminus of TRPA1. Strikingly, structurally unrelated cystein modifying reagents such as N-methyl maleimide (NMM), iodoacetamide (IA), and (2-aminoethyl)methanethiosulphonate (MTSEA) also activated TRPA1 (Hinman et al., 2006; Macpherson et al., 2007). Thus, a common activation mechanism involving cysteine modifications may explain the existence of surprisingly large amount of TRPA1 agonists.

1.4.4.2 Activation by noxious cold

The cold sensitivity of TRPA1 is in extensive debate. When Patapoutian and colleagues cloned TRPA1, they found that TRPA1 expressed in *Xenopus* oocytes and CHO cells was activated by noxious cold temperature with an activation threshold of 17ºC (Story et al., 2003). Similar results were reported from the same group in 2004 (Bandell et al., 2004). However, Julius’s group was not able to reproduce the noxious cold sensitivity of TRPA1 either heterologously expressed in their HEK293 cells or endogenously expressed in cultured rat trigeminal neurons (Jordt et al., 2004). Human feelings to TRPA1 agonists are also complex. Super cooling compound icillin activates TRPA1 but it also activates cold receptor TRPM8. All other known TRPA1 agonists typically do not cause cooling sensation. In contrast, some of them cause burning sensation. Recently, two distinct strains of TRPA1 knockout mice were generated by two independent groups. Julius’ group found that TRPA1 deficient mice showed normal cold sensitivity (Bautista et al., 2006), whereas Corey’s group showed
that TRPA1 knockout mice displayed a significant defect in cold sensation to an
extreme low temperature-0°C (Kwan et al., 2006).

1.4.4.3 Activation by receptors

Although debates exist regarding the cold sensitivity of TRPA1, it is widely
accepted that TRPA1 is operated by receptors (Jordt et al., 2004; Bandell et al.,
2004). It was shown that the activation of Gq/11-coupled receptors such as
muscarinic receptor M1R and bradykinin receptor B2R led to the activation of
TRPA1 (Jordt et al., 2004; Bandell et al., 2004). Ca²⁺ release from ER store was
able to activate TRPA1 without the requirement of other factors (Jordt et al.,
2004). Recently, it was confirmed that intracellular Ca²⁺ could directly activate
TRPA1 by binding to an intrinsic Ca²⁺-binding motif (EF-hand) within the C-
terminus of TRPA1 (Zurborg et al., 2007).

1.4.4.4 Activated by mechanic force

TRPA1 is highly expressed in hair cells of mammalian inner ear, where it was
suggested to function as a hearing transduction channel. Indeed, knockdown of
mouse TRPA1 by siRNA or zebra fish TRPA1 by morpholino oligonucleotides
greatly inhibited hearing transduction in these animals (Corey et al., 2004).
However, two strains of TRPA1 knockout mice as discussed above disputed the
essential role of TRPA1 in hearing. Both studies found that TRPA1 knockout
mice displayed normal hearing capability (Bautista et al., 2006; Kwan et al.,
2006). Given that TRPA1 is highly expressed in inner ear and multiple protein-
protein interaction ankyrin-like domains are present within the N-terminus of TRPA1, it is still possible that although TRPA1 does not form a hearing transduction channel by itself, it plays a regulatory role by interacting with other channel-forming proteins. Interestingly, a *C. elegans* TRPA1 homolog was shown to be mechano-sensitive in both *C. elegans* sensory neurons and mammalian expression system (Kindt et al., 2007). More detailed studies are required to establish the mechanosensitivity of mammalian TRPA1.

1.4.5 Activation and regulation of TRPP channels

No direct agonist has been identified for TRPP subfamily channels. TRPP2 is the best studied member within this group of TRP channels. TRPP2 was shown to be expressed in the primary cilia of kidney epithelia cells and involved in the mechanic flow-induced Ca\(^{2+}\) influx pathway (Nauli et al., 2003). Therefore, Nauli et al have proposed that TRPP2 is a mechanic-sensor in the primary cilia of kidney collecting duct epithelia cells (Nauli et al., 2003). However, more studies are required to establish the mechano-sensitivity of TRPP2. TRPP2 was also shown to be mainly localized intracellularly and to function as an ER Ca\(^{2+}\)-release channel (Koulen et al., 2002). TRPP1 was able to directly interact with TRPP2, which may help the translocation of TRPP2 from ER to plasma membrane and in order for it to function as a plasma membrane Ca\(^{2+}\) permeable cation channel (Hanaoka et al., 2000).

1.4.6 Activation and regulation of TRPN channels
TRPN channels are most likely mechano-sensitive in *Drosophila*, *C. elegans*, and zebra fish. NOMPC channel in *Drosophila* has been shown to be expressed in mechanosensory organs and the deletion of this channel completely abolished *Drosophila* mechanosensory transduction current (Walker et al., 2000). In zebra fish, TRPN1 was shown to encode the hearing transduction channel in sensory hair cells of inner ear (Sidi et al., 2003). Knockdown of TRPN1 in zebra fish by morpholino injection caused loss of electric transduction in hair cells and deafness (Sidi et al., 2003). TRP4, the *C. elegans* homolog of *Drosophila* NOMPC, was also shown to be expressed in mechanosensitive DVA neuron and responsible for the stretch-induced response in *C. elegans* (Li et al., 2006). The above lines of evidence from different species have confirmed the mechanical gating mechanism of TRPN channels. However, a detailed molecular picture on the mechanosensitity of TRPN channel remains elusive.

1.4.7 Activation and regulation of TRPML channels

Although the general role of TRPML channels might be related to the normal lysosomal biogenesis and maintenance, little work has been carried out regarding the activation mechanism of these channels. When heterologously expressed, TRPML channels, particularly TRPML3, typically showed very large constitutive current (Kim et al., 2008; Xu et al., 2007). This constitutive current was inhibited by low pH and three putative histidines in the extracellular loop between S1 and S2 were involved in the proton-mediated inhibition (Kim et al., 2008).
1.5 Aims of the studies and summary of the findings

Compared to other cation channels such as voltage-gated Ca\(^{2+}\) channels, voltage-gated Na\(^+\) channels, and voltage-gated K\(^+\) channels, the history of the investigation of TRP channels is still short and large part of functional and structural features for TRP channels are still unknown. Many types of voltage-independent cation influx exist in different types of tissues and cells, but their molecular identity remains elusive (Nilius et al., 2007). TRP superfamily channels are one of the best candidates for these cation influx pathways because their ubiquitous expression patterns, polymodal sensor feature, and voltage-independent gating mechanisms. Moreover, several TRP channels have been clearly shown to be essential for temperature sensing and pain sensation, two fundamentally important functions for all mammals including human beings. Therefore, there is a good reason for us to devote to the study of the structure and function of TRP channels.

The major aim of this thesis is to add some useful information regarding the activation and regulation mechanisms of TRP channels into the TRP community. I focused on three TRP channels: TRPV3 in the TRPV subfamily, and TRPC4 and TRPC5 in the TRPC subfamily. With the help of my advisor Dr. Michael X. Zhu and other colleagues, I have studied and discovered that:

1): Multiple poly-unsaturated fatty acids (PUFAs) were able to greatly potentiate TRPV3.
2): $\text{Ca}^{2+}$ was important for the sensitization of TRPV3 upon repetitive stimulation.

3): A naturally occurring point mutation of TRPV3 associated with hair-loss in rodents caused constitutive activation of TRPV3 channel and host cell death.

4): TRPC4 and TRPC5 are integrators of two distinct G protein signaling pathways.
Figure 1.1 Phylogenetic tree of mammalian TRP channels.
All 28 mammalian TRPs are shown in 6 subfamilies with TRPC2 being a pseudogene in human: most TRPC channels form store operated Ca\(^{2+}\) channels (SOC) or receptor operated Ca\(^{2+}\) channels (ROC); TRPV1-V4 are directly activated by heat with distinct temperature thresholds; TRPM2, M4, and M5 are also heat-activated channels in the presence of intracellular co-factors; TRPM8 is directly activated by cooling temperatures; and TRPA1 is possibly activated by noxious cold temperatures. (PAM stands for Percent Accepted Mutation)
Figure 1.2 General architecture of TRP channels.

A, Hydrophobicity plot of a TRP channel (TRPC3). Although 8 hydrophobic regions exist, only 6 of them are thought to form transmembrane segments (S1-S6).

B, Proposed topology of TRP channels. Similar to voltage-gated K+ channel, both N- and C-termini of TRP channels are intracellular, and the S5-S6 loop forms the pore loop.

C and D, TRP channel is composed of 4 channel subunits and these subunits can be either identical or distinct to form homotetramer or heterotetramer.
Figure 1.3 Some important binding partners for TRP channels.

Common features for each TRP subfamily are shown: TRP domains immediately following S6 are present in all TRPC, TRPM, and TRPN channels; a common CaM and IP3R binding site in the C-terminus exists in all TRPC channels; multiple ankyrin repeats are localized in the N-termini of all TRPC, TRPV, TRPA, and TRPN channels.
CHAPTER 2

MATERIALS AND METHODS

2.1 Reagents and DNA constructs

All chemicals were purchased from Sigma-Aldrich or Fisher Scientifics except otherwise mentioned. Linoleic acid, docosahexaenoic acid, α-linolenic acid, glinolenic acid, 5,8,11,14-eicosatetraynoic acid (ETYA), nordihydroguaiaretic acid (NDGA), and 2APB were purchased from Cayman Chemical Inc. (Ann Arbor, MI). Rev-5901, baicalein, cinnamyl-3,4-dihydroxy-cyanocinnamate (CDC), esculetin, 17-octadecynoic acid (17-ODYA), and SKF525A were purchased from BioMol (Plymouth Meeting, PA). 4-[(2S)-2-[(5-isoquinolinylsulfonyl)methylamino]-3-oxo-3-(4-phenyl-1-piperazinyl)propyl]phenyl isoquinolinesulfonic acid ester (KN-62) and DAMGO were purchased from Tocris Cookson Inc. (Ellisville, MO). Ro318220 and Go6976 were purchased from EMD Bioscience (La Jolla, CA). Lipofectamine 2000, Opti-MEM, Fluo4/AM, Pluronic F-127, thapsigargin, blasticidin-S, zeocin, and all cell culture reagents were purchased from Invitrogen (Carlsbad, CA). Fura2/AM was purchased from TEFLabs (Austin, TX). T7 mMessage mMachine cRNA synthesis kit was purchased from Ambion (Austin, TX). TNT-coupled rabbit reticulocyte system was purchased from Promega.
Collagenase was purchased from Worthington Biochem. (Madison, WI). FLIPR-membrane potential dye-blue was purchased from Molecular Devices (Sunnyvale, CA). CaM-Agorose and all western blotting reagents were purchased from GE Healthcare (Waukesha, WI). Pertussis toxin (PTX) was purchased from List Biological Lab (Campbell CA).

The following mammalian expression vectors were used to accommodate various complementary DNAs (cDNAs) of TRP channels: pcDNA3 and pcDNA4 T/O from Invitrogen (Carlsbad, CA), pIRESneo, pIREShyg2, pIRESEGFP, and pEGFPC3 from Clontech (Mountain View, CA), pIRESZeocin was created in the lab. PCR-based site-directed mutagenesis was performed according to the protocol provided by Stratagene (La Jolla, CA).

2.2 Mammalian cell culture and transfection

Human embryonic kidney 293 (HEK293) cells and T-Rex 293 cells (Invitrogen) were grown at 37°C, 5% CO₂ in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), 50 units/ml penicillin, and 50 μg/ml streptomycin. Specific antibiotics, including zeocin, hygromycin-B, and G-418, were added into regular culture medium for selecting and maintaining cells stably expressing pIRES-Zeocin, pIRES-hyg2, and pIRES-neo containing constructs, respectively. For T-Rex 293 cells, blasticidin S (10 μg/ml) was included. Mouse 308 keratinocytes were grown in a 3:1 (v/v) mixture of Dulbecco’s minimal essential medium and Ham’s F-12 medium supplemented
with 10% heat-inactivated fetal bovine serum, 5 mg/ml insulin, 0.4 mg/ml hydrocortisone, 5 mg/ml transferrin, 2 nM 3,3'-50 triiodo-L-thyroxine, 0.1 nM cholera toxin, 10 ng/ml epidermal growth factor, 60 mg/ml penicillin, and 25 mg/ml gentamicin as described by Chung et al. (2003).

Lipofectamine 2000 was used in most transfection experiments except otherwise mentioned. Standard transfection was performed based on the protocol provided by the manufacturer. Briefly, HEK293 cells with about 90% confluency were detached from cell culture dishes by trypsin digestion and used for transfection experiments. Most transfections were performed in 96-well plates pre-coated with 20 μg/ml polyornithine, with each well containing a 50 μl transfection mixture: 0.1 μg desired DNA and 0.3 μl lipofectamine 2000 were mixed in 50 μl Opti-MEM. Transfection mixture was put in wells of 96-well plate at room temperature for about 30 min before the addition of 100 μl resuspended HEK293 cells with a cell density of about 10^6/ml. Cells with transfection mixture were maintained in tissue culture incubator for 1-2 days without medium change. A typical >70% transfection efficiency could be achieved judged by the number of green cells transfected with enhance green fluorescent protein (EGFP) coding sequence-containing DNA constructs. Depending on usage, transfected cells in 96-well plate were either split and re-seeded into 35mm culture dish for patch clamp experiments or directly used for Ca^{2+} and membrane potential measurement.

2.3 Immunodetection of TRP channels
TRPC5-transfected cells were incubated with 1.5 ml PBS containing 0.5 mg/ml Sulfo-NHS-LC biotin on ice for 30 min. Biotinylation process was terminated by 50 mM glycine. After lysis in RIPA buffer, aliquots of crude lysates were incubated with streptavidin-agarose beads for 120 min. The beads were washed and resuspended in 2x SDS-PAGE loading buffer. Biotinylated membrane proteins and total proteins were subjected to SDS-PAGE with 8% gel and the presence of TRPC5 protein was detected by western blotting using a polyclonal anti-TRPC5 antibody.

2.4 cRNA synthesis and expression in Xenopus oocytes

Full-length mouse TRPV3 and its mutants were constructed into a T7 promoter-containing vector PAGA3. XhoI flanking the C-terminus of TRPV3 was used to linealize DNA. T7 mMessage mMachne cRNA synthesis kit was used to synthesize the complementary RNAs (cRNAs). Purified cRNAs were quantified by spectrophotometer and diluted with nuclease free water into 100 ng/ml.

Oocytes from sexually mature *Xenopus laevis* of older than 2.5 years (Xenopus I, Inc., Dexter, MI) were used for cRNA injection and expression. All experiments using *Xenopus* were performed according to the protocols approved by OSU Institutional Animal Care and Use Committee (IACUC). Briefly, frogs were anesthetized with 0.1% ethyl 3-aminobenzoate methanesulfonate at 0°C for about 15 minutes, and part of ovarian lobe was removed and cut into small pieces. Then the lobe pieces were washed three times in OR2 solution containing
(in mM): NaCl 82.5, KCl 2, MgCl2 1, HEPES 5, pH7.4. OR2 solution supplemented with 1 mg/ml collagenase (Worthington Biochem, Lakewood, NJ) was used to digest lobe pieces for 90 min in a 19 °C air shaker. Denuded, healthy-looking oocytes of more than 1 mm in diameter were selected by a dissection microscope and maintained in a 19°C incubator for later cRNA injection.

Typically, one day after incubation, dissociated healthy *Xenopus* oocytes were used for cRNA injection. Each oocyte was injected with about 50 nl nuclease-free water containing 5 ng desired cRNA. Injected oocytes were returned to 19 °C incubator and maintained in sterile ND96 solution containing (in mM): NaCl 96, KCl 2, CaCl2 1.8, MgCl2 1, HEPES 5, pH 7.6, supplemented with 275 μg/ml pyruvic acid and 20 μg/ml gentamycin. ND96 solution was changed daily.

2.5 Membrane potential and intracellular [Ca2+] measurements by the FlexStation

When reaching about 95% confluency, transient or stably transfected HEK293 cells in 96-well plates were washed three times with regular extracellular solution (ECS) containing (in mM): NaCl 140, KCl 5, CaCl2 2, MgCl2 1, glucose 10, HEPES 15, pH 7.4, then incubated in 60 μl ECS supplemented with 2 μM fluo4/AM, 2 mM probenecid, and 0.1% Pluronic F-127 at 37°C in darkness for about 45 min. After incubation, cells were washed three times and placed in 80 μl ECS. An automated florescence plate reader integrated with a robotic 8-channel fluid handling system, Flex Station (Molecular Devices, Sunnyvale, CA) was used
to monitor the intracellular [Ca\textsuperscript{2+}]. The Fluo4 fluorescence was measured at excitation of 494 nm and emission of 525 nm from the bottom of the plate at 0.67 Hz.

For membrane potential measurement, each vial of FLIPR membrane potential dye-blue was diluted into 10 ml ECS and stored as stock dye solution at -80ºC until use. Before experiment, the stock dye solution was freshly defrosted and further diluted four fold in ECS (loading solution). For membrane potential dye loading, 80 \( \mu \text{l} \) of dye loading solution was added into each test well. After about 30 min incubation at room temperature, membrane potential of cells was measured at excitation of 535 nm and emission of 565 nm according to the protocol provided by Molecular Device.

2.6 Intracellular [Ca\textsuperscript{2+}] measurement by the PTI-DeltaRam Ca\textsuperscript{2+} imaging workstation

Cells were seeded on 13-mm round coverslips pre-coated with polyornithine one day before experiment. During experiment, cells on coverslip were washed once with the same regular ECS as for the Flex-station experiments, and then incubated in 500 \( \mu \text{l} \) ECS supplemented with 0.1% bovine serum albumin (BSA), 2 \( \mu \text{M} \) fura2/AM and 0.05% Pluronic F-127 at 37ºC in darkness for 30 min. At the end of incubation, Fura2/AM loaded cells on coverslip were washed three times with regular ECS and the coverslip was transferred to a [Ca\textsuperscript{2+}] imaging chamber
(Warner Instruments, Hamden, CT) for the following single-cell intracellular $[Ca^{2+}]$ measurement.

Intracellular $[Ca^{2+}]$ of a group of about 20 cells was measured ratiometrically by Fura2 using a PTI (Photon Technology International, Lawrenceville, NJ) photometry system attached to a Nikon Eclipse TE200 inverted microscope. The Fura2 fluorescence was measured at the alternating excitation wavelengths of 340 and 380 nm and the emission of 510 nm.

2.7 Whole-cell recordings of HEK293 cells and T-Rex293 cells

Transfected cells (either transient or stable) were reseeded in 35-mm dishes one day after the transfection. Whole-cell recordings were performed in the following 1-2 days. Recording pipettes were pulled from micropipette glass (World Precision Instruments Inc, Sarasota, FL) and fire polished. The resistance of pipettes was 1.5–4 MΩ when filled with pipette solution. In most cases, regular ECS was used for diluting chemicals and perfusing cells extracellularly unless otherwise mentioned. Isolated single cells were voltage-clamped in the whole cell mode using an EPC9 (HEKA Instruments Inc, Southboro, MA) amplifier. Voltage commands were made from the Pulse+Pulse Fit program (version 8.53, HEKA), and the currents were recorded at 5 kHz.

Two voltage protocols were typically used to study TRP channels: ramp protocol and step-pulse protocol. In the ramp protocol, voltage ramps of 100 ms to $+100$
mV after a brief (20 ms) step to -100 mV from holding potential of 0 mV were applied every 0.5 s. In the step-pulse protocol, 16 episodes of 300-ms step pulses start from -100 mV with a 20-mV increment. Each step pulse was followed by a 250-ms step to −100 mV, giving rise to tail currents. The cells were continuously perfused with the bath solution through a gravity-driven multi-outlet device with the desired outlet placed about 50 µm away from the cell being recorded. Chemicals were dissolved with in concentrations in appropriate solvents as stock solutions and stored at -20°C or -80 ºC. Before experiments, chemical stocks were diluted in the appropriate external solutions to the desired final concentrations and applied to the cell through perfusion. All whole-cell experiments were performed at the room temperature (20–24°C) unless otherwise mentioned.

2.8 Two-electrode voltage clamp recordings of *Xenopus* oocytes

Desired cRNA-injected and healthy-looking oocytes were placed in a RC-3Z oocyte recording chamber (Warner Instruments, Hamden, CT) and perfused with a bath solution that contained (in mM): NaCl 100, KCl 2.5, MgCl₂ 1, Hepes 5, pH 7.4. The oocytes were impaled with two intracellular glass electrodes (filled with 3M KCl) connected to an OC-725C Oocyte Clamp amplifier (Warner Instruments). Voltage commands were made from the Pulse+Pulsefit program (HEKA Instruments, Southboro, MA) via an ITC-18 Computer Interface (Instrutech Co. Port Washington, NY). Oocytes were clamped at −20 mV, stepped to −100 mV for 20 ms, followed by a voltage ramp of 200 ms from −100 to +100 mV once
every 0.56 s. Currents were recorded at the sampling rate of 1 kHz. Temperature changes were achieved by using a CL-100 bipolar temperature controller connected to a SC-20 dual in-line solution heater/cooler (Warner Instruments).

2.9 CaM binding assay

A series of TRPV3 cDNA segments were generated by restriction digestion or PCR. Then these fragments were fused to the C-terminus of maltose-binding protein (MBP). The encoded peptides were synthesized \textit{in vitro} in the presence of $[^{35}\text{S}]$methionine using TNT-coupled rabbit reticulocyte lysate system (Promega), then incubated with CaM-agarose in a CaM-TRP binding buffer containing (in mM): KCl 120, 0.5% Lubrol, Tris-HCl 20, pH 7.5, and with the absence or presence of 50 μM free Ca$^{2+}$ buffered with 10 mM HEDTA. The amount of peptide retained by CaM-agarose was revealed by autoradiography after separation of the washed products by SDS-PAGE.
CHAPTER 3

UNSATURATED FATTY ACIDS POTENTIATE TRPV3

The main discovery presented in this chapter has been published in 2006 (Hu et al., 2006). Three equal contributors including Hong-zhen Hu, Rui Xiao, and Chunbo Wang performed most experiments for this chapter. Specifically, figure 3.1 was provided by Dr. Chunbo Wang (Dr. Wang also prepared all cRNAs for oocyte recordings); figure 3.2, 3.5, 3.6, 3.7, and 3.8 were provided by Dr. Hong-zhen Hu; figure 3.3 and 3.4 were provided by Rui Xiao. This represents the nature of the collaboratory research in our laboratory.

3.1. Introduction

TRPV3 was cloned by three independent groups using homology cloning strategy based on TRPV1 sequence (Xu et al., 2002; Smith et al., 2002; Peier et al., 2002b), and the assumption was that TRPV3 should share some sequence homology as well as functional similarity to the founding member of the TRPV subfamily, TRPV1. Indeed, the overall sequence of mouse TRPV3 is about 43% identical to TRPV1 and TRPV4, and similar to TRPV1 and TRPV4, TRPV3 is activated by heat with a temperature threshold of about 33°C (Peier et al., 2002b).
TRPV1 has been repeatedly shown to be sensitive to many inflammatory mediators, such as bradykinin, nerve growth factor, histamine, and ATP through signaling pathways mediated by activation of their respective receptors (Chuang et al., 2001; Tominaga et al., 2001; Kim et al., 2004) and by tissue acidosis as a consequence of inflammation and malignant tumor growth (Reeh and Kress, 2001). On the other hand, studies with TRPV4 knockout mice have supported the important role of TRPV4 in mediating pain-related behavior induced by mild hypertonic stimuli in the presence of inflammatory mediators (Alessandri-Haber et al., 2005). The expression level of another homolog of TRPV3, TRPV2, was also shown to be up-regulated in DRG neurons by peripheral inflammation (Shimosato et al., 2005). Given that TRPV3 is highly expressed in skin keratinocyte and it shares sequence and functional similarities to TRPV1, TRPV2, and TRPV4, it is reasonable to speculate that TRPV3 also plays a role in inflammatory responses during acute tissue injury.

Arachidonic acid (AA) and its metabolites are important mediators of inflammation (Wenzel et al., 1997). Under resting conditions, AA is stored in the plasma membrane of virtually all cells in forms of phospholipids. During inflammation process, AA is released from the infiltrating lymphocytes or local sensory fibers and skin cells in response to other inflammatory mediators such as histamine, bradykinin, ATP, and NGF. The generation of AA from phospholipids in plasma membrane occurs through three pathways: 1)
phospholipase D (PLD) initiated conversion of phosphatidyl-ethanolamine or phosphatidyl-choline to phosphotidic acid and subsequently AA; 2) PLC initiated conversion of phosphatidylinositol to DAG and AA by DAG lipase; and 3) direct action of phospholipase A2 (PLA2) to convert phospholipids to AA. Interestingly, both TRPV1 and TRPV4 have been shown to be activated by AA or its metabolites, namely, lipoxygenase products of AA directly activate TRPV1 (Hwang et al., 2000; Shin et al., 2002), and the epoxygenase products are responsible for the stimulatory effect of AA on TRPV4 (Watanabe et al., 2003). However, the effect of AA and its lipid metabolites on TRPV3 was not studied before, therefore, we performed a detailed study on it.

3.2. AA potentiates TRPV3 mediated \([\text{Ca}^{2+}]_{i}\) elevation

To study the potential effect of AA on TRPV3, we first performed single cell intracellular \(\text{Ca}^{2+}\) imaging experiments. It has been reported that TRPV3 was highly expressed in skin keratinocytes (Chung et al., 2004), and 2-aminoethoxydiphenyl borate (2-APB), a common activator for TRPV1/V2/V3, was able to activate TRPV3 endogenously expressed in keratinocytes (Chung et al., 2004). In Fura-2 loaded mouse 308 keratinocytes, 300 \(\mu\)M 2-APB induced a small \([\text{Ca}^{2+}]_{i}\) increase (Fig 1A), and the co-application of AA with 2-APB greatly potentiated \([\text{Ca}^{2+}]_{i}\) increase in a dose-dependent manner (Fig 1A).

TRPV4 is also expressed in keratinocytes (Chung et al., 2004) and the epoxygenase products of AA are able to activate TRPV4 (Watanabe et al., 2003).
To rule out the involvement TRPV4 in the AA-induced potentiation effect observed in mouse 308 keratinocytes, similar Ca\(^{2+}\) imaging experiments were performed using HEK293 cells transiently transfected with mouse TRPV3. These cells do not express endogenous TRPV3 or TRPV4. AA greatly potentiated [Ca\(^{2+}\)] \text{increase} induced by 100 \(\mu\)M 2-APB (Fig 1B). In contrast, the [Ca\(^{2+}\)] \text{rise} induced by AA itself was very small and has a much slower kinetics compared with that induced by 2-APB, and more importantly, it lacked a dose-dependence for AA-induced activation (Fig 1C). Therefore, it is most likely that AA potentiates the [Ca\(^{2+}\)] \text{increase} through regulating TRPV3.

3.3. AA potentiates 2-APB induced TRPV3 currents

[Ca\(^{2+}\)] is sensitive to various intracellular and extracellular factors, and sometimes a [Ca\(^{2+}\)] increase might result from an indirect effect of channel opening (for example, the opening of voltage-gated Na\(^+\) channels depolarizes cells which in turn opens high-voltage-gated Ca\(^{2+}\) channels and induces a [Ca\(^{2+}\)] increase, and this [Ca\(^{2+}\)] increase is an indirect effect of the opening of voltage-gated Na\(^+\) channels). In order to confirm the potentiation effect of AA on TRPV3, we performed whole-cell recordings using TRPV3-transfected HEK293 cells. Outwardly-rectifying TRPV3 current was evoked by 100 \(\mu\)M 2-APB and a slow sensitization process was observed by repetitive 2-APB treatment (Fig 3.2A). However, in the presence of 10 \(\mu\)M AA, a 4-fold potentiation of TRPV3 current at both -100 mV and +100 mV occurred, which is much larger than the potentiation induced by repetitive 2-APB stimulation alone (Fig 3.2A). Importantly, we
performed similar whole-cell recordings with TRPV1- and TRPV2-transfected HEK293 cells, no potentiation effect were found for 100 μM 2-APB evoked TRPV1 or TRPV2 currents (Fig 3.2 C and E), indicating that the potentiation effect of AA is specific for TRPV3.

3.4. Functional properties of AA-potentiated TRPV3 current

Ruthenium red (RR), a positively charged non-specific blocker for many TRP channels, has been shown to directly bind to the conserved Asp647 within the putative pore-loop of TRPV1, which blocks the entry of extracellular cations (García-Martínez et al., 2000). TRPV3 has a counterpart residue residing in its putative pore-loop-Asp641. The AA-potentiated TRPV3 inward current was completely blocked by 10 μM RR; however, the outward current of TRPV3 was increased during RR treatment (Fig 3.3A), indicating that although the binding of RR to TRPV3 pore blocks the entry of extracellular cations, it somehow facilitates the efflux of intracellular cations. Importantly, both the inhibitory effect of RR for TRPV3 inward current and stimulatory effect for TRPV3 outward current were not affected by AA (Fig 3.3 C and D).

We also studied the dose dependence of AA for the potentiation of TRPV3. During a continuous application of 100 μM 2APB, a gradient of increasing concentration of AA was applied (Fig 3.3 E). By normalizing all responses to the maximal response induced by 100 μM 2APB and 100 μM AA, the EC50 values of AA were estimated to be 21.9±4.5 μM (Hill coefficient, n_H=1.2±0.5) at +100mV.
and 22.6±2.5 μM (n_1=1.5±0.3) at -100 mV (Fig. 3G). At high concentrations, AA may form miscells that disrupt plasma membranes and cause a non-specific leak. However, this is not an issue in our study because the preincubation of the highest AA concentration used, 100 μM, for 30 sec did not cause any leak-current, whereas the preincubation greatly potentiate the response to 100 μM 2APB (Fig 3.3 H).

3.5. Single channel recordings of AA-potentiated TRPV3 currents in HEK293 cells
AA can be generated from both endogenous and exogenous sources. When released endogenously, the sites of action for AA may be mainly intracellular; when released from neighboring cells, the sites of action could be either intracellular or extracellular because AA is membrane permeable. In order to determine the sidedness of AA action, we performed single channel recordings with excised inside-out and outside-out patches. In TRPV3-transfected, but not vector-transfected, control HEK293 cells, 5-10 μM 2APB was able to activate TRPV3. Interestingly, in the presence of 10 μM AA in both inside-out and outside-out configurations, the open probability of TRPV3 was significantly increased compared to 2APB treatment alone, with a increments of 9.1±3.9 fold (n=7) (Fig 3.4 A and D) and 4.8±1.0 fold (n=4) (Fig 3.4 B, C and E) for inside-out and outside-out patches, respectively. From the single channel recordings we could not draw a conclusion on the sidedness of AA action; however, it indicates
that the potentiation effect of AA could arise either in autocrine or in paracrine manner, which is consistent with the finding that 12-HPETE, an endogenous TRPV1 lipid agonist, activates TRPV1 both in antocrine and paracine manners (Sexton et al., 2007; Gibson et al., 2008).

3.6. AA potentiates 2APB-induced TRPV3 current in Xenopus oocytes

Xenopus oocyte expression system is an alternative system to study ion channels. By comparing the results from HEK293 expression system and Xenopus oocyte expression system, we can obtain useful information on whether the ion channel regulation is dependent on the host cell type. Another advantage of using Xenopus oocytes to study TRPV3 is that TRPV3 expressed in Xenopus oocytes does not display an obvious sensitization process upon repetitive stimulation, which is typically observed in mammalian cell expression systems (Xu et al., 2002; Peier et al., 2002b). This helps rule out the possibility that the potentiation effect of AA observed above was due to the intrinsic potentiation of TRPV3 expressed in HEK293 cells.

Similar to the results in HEK293 cells, after cRNA injection in Xenopus oocytes 10 μM AA greatly potentiated TRPV3 (Fig 3.5 A), but not TRPV1 (Fig 5.3 C), current evoked by 100 μM 2APB. On the other hand, anandamide (AEA), an agonist of TRPV1, activated TRPV1 in a synergistic manner with 2APB (Fig 3.5 C) but only slightly potentiated 2APB-evoked TRPV3 current (possibly due to a
partial conversion of AEA to AA (Watanabe et al., 2003)). Therefore, the potentiation effect of AA is specific to TRPV3.

3.7. Dose-dependence of AA-potentiated TRPV3 current

Similar to the experiments with TRPV3-expressing HEK293 cells, the dose-dependence of AA-potentiated TRPV3 current was studied in *Xenopus* oocytes. AA at a concentration as low as 10 μM was able to obviously potentiated 2APB (100 μM)-evoked TRPV3 current in *Xenopus* oocytes (Fig 3.6 A). The EC<sub>50</sub> value for the potentiation effect of AA was determined to be 7.1±0.6 μM (Hill coefficient, n<sub>H</sub>=1.2±0.1) at +100 mV and 8.5±0.5 μM (n<sub>H</sub>=1.4±0.1) at -100 mV (Fig 3.6 B).

To further characterize the potentiation effect of AA on 2APB-evoked TRPV3 current, we established the dose response curve of 2APB for TRPV3 expressed in *Xenopus* oocytes in the absence or presence of 10 μM AA. Notably, the EC<sub>50</sub> value of 2APB alone for TRPV3 expressed in *Xenopus* oocytes was determined to be 1060±42 μM (n<sub>H</sub>=1.7±0.2) (Fig 3.6 C and D), which was roughly 10-fold higher than the value determined in the HEK293 expression system (Hu et al., 2004; Chung et al., 2004). This EC<sub>50</sub> difference between expression host systems was also observed for capsaicin: 0.26±0.06 μM for TRPV1 expressed in HEK293 cells (Wang et al., 2004) and 1.9±0.2 μM for TRPV1 expressed in *Xenopus* oocytes (McIntyre et al., 2001). The dose-response curve of 2APB for TRPV3 expressed in *Xenopus* oocytes was left shifted and the EC<sub>50</sub> value was
calculated to be 348±37 μM (n=1.2±0.1) in the presence of 10 μM AA (Fig 3.6 C and D).

3.8. TRPV3 is directly potentiated by AA but not its downstream molecules

AA potentiates TRPV3 both endogenously expressed in mouse 308 keratinocytes and exogenously expressed in HEK293 cells and Xenopus oocytes. Because TRPV1 and TRPV4 are sensitive to the metabolism products of AA, we used various AA metabolism blockers to study the involvement of AA metabolites in the potentiating effect of AA: including cyclooxygenase inhibitor piroxicam (60 μM), lipoxygenase inhibitors NDGA (10 μM), 5,8,11-eicosatriynoic acid (ETI, 10 μM), Rev-5901 (10 μM), baicalein (10 μM), CDC (10 μM), and esculetin (10 μM), and epoxygenase inhibitors 17-ODYA (10 μM), miconazole (10 μM), and SKF525A (10 μM), as well as the nonmetabolizable AA analogue ETYA (1–100 μM). None of above drugs blocked the potentiation effect of AA to the 2APB-evoked TRPV3 current in Xenopus oocytes (Fig 3.7 C-F). Surprisingly, CDC, ETI, ETYA, and NDGA by themselves potentiated 2APB-evoked TRPV3 current. Particularly, ETYA, a triple-bond analog of AA, and ETI, a triple-bond analog of another polyunsaturated fatty acid, 5,8,11-eicosatrienoic acid (20:3 n9, mead acid), also strongly potentiated TRPV3 (Fig E and F), indicating that TRPV3 is directly regulated by free fatty acids but not their oxidation products.
AA and its derivatives also activate many other intracellular signaling pathways, such as PKC/mitogen-activated protein (MAP) kinase involved pathway (Barry et al., 1999), and Ca²⁺/CaM involved pathway (Muthalif et al., 2001). We tested PKC inhibitors Ro318220 (1 μM) and Go6976 (0.3 μM), CaM-dependent kinases inhibitor KN-62 (3 μM), and PLC inhibitor U73122 (10 μM), none of them had any effect on the potentiation of AA to TRPV3 (Fig 3.7 A and B). Most likely, AA regulates TRPV3 without the involvement of its downstreaming signaling pathways.

3.9. Other unsaturated fatty acids also potentiates TRPV3
Given that nonmetabolizable AA analog ETYA also potentiated TRPV3, we reasoned that it was possible that fatty acids, in general, potentiated TRPV3. To test this hypothesis, we studies the putative potentiating effect of a number of fatty acids, including a saturated fatty acid, palmitic acid (16:0), trans unsaturated fatty acids, elaidic (trans 18:1 n9) and linoelaidic (all trans 18:2 n6) acids, and many cis unsaturated fatty acids of the Δ9, ω-3, and ω-6 desaturase families. Surprisingly, saturated fatty acids such as palmitic acid up to 500 μM had no effect on 2APB-evoked TRPV3 current (n=3, data not shown), while all other unsaturated fatty acids at 10 μM were able to potentiate TRPV3 with different potency, implying that the potentiating effect of fatty acids are restricted to unsaturated fatty acids (Fig 3.7 A). We also noticed that for PUFAs, the positions of double bonds seem to determine the potency of the potentiation effect. For
example, all PUFAs with the first double-bond starting at the fifth position from
the carboxylated head group, such as 5,8,11-eicosatrienoic acid (20:3 n9),
5,8,11,14-eicosatetraenoic acid (AA, 20:4 n6), and 5,8,11,14,17-
eicosapentaenoic acid (20:5 n3) (structure shown in Fig 3.7 B), showed the
strongest potentiation effect of about 10-fold (Fig 3.7 A). However, the molecular
mechanism for this phenomenon requires further investigation.

3.10. Summary

The important role of TRPV1 in the mammalian inflammatory response and pain
sensation has been firmly established (Julius & Basbaum, 2001; Okajima &
Harada, 2006). Multiple molecular mechanisms have been proposed and
confirmed for the up-regulation of TRPV1 function by various inflammatory
mediators. Among these mediators, some directly activate TRPV1 such as
protons (Tominaga et al., 1998) and 12-HPETE (Gibson et al., 2008), a derivative
of AA; some release TRPV1 from the inhibition of PIP2 by activating their
respective Gq/11-coupled receptors and breaking down PIP2 such as bradykinin
and histamine (Chuang et al., 2001); some potentiate TRPV1 channel function by
phosphorylation through PKA or PKC signaling pathway such as some proteases
released during inflammation and injury (Dai et al., 2004); and some foster the
plasma membrane expression level of TRPV1 by promoting channel
translocation such as NGF (Stein et al., 2006).
Similar to TRPV1, TRPV3 might also play a role in inflammatory response. We found that the activation of TRPV3 was significantly potentiated by AA and other PUFAs in both native cell type and heterologously expressed cell type. AA is an important mediator of inflammation, and it is released in both autocrine and paracrine fashions during inflammation and acute tissue injury. Our finding that TRPV3 was potentiated by both extracellular and intracellular AA is consistent with the activation of TRPV1 by both extracellular and intracellular 12-HPETE. Given that unlike TRPV1, TRPV3 has not been shown to be sensitive to protons, NGF, bradykinin and other inflammatory factors; our results provide a mechanism for TRPV3 up-regulation. During inflammation, TRPV3 in skin and peripheral neurons is functionally up-regulated by AA and other PUFAs, which may be partially responsible for hyperalgesia typically found during tissue injury and inflammation.

Interestingly, the potentiation effect by AA does not require any metabolism of AA or major signaling pathways related to AA, which is different from TRPV1 and TRPV4. Therefore, it is mostly likely that PUFAs either directly bind to TRPV3 and regulate its gating or change some plasma membrane properties and somehow modify the channel opening probability. Noticeably, for *Drosophila* TRP and TRPL, various PUFAs were shown to directly activate the channels without the involvement of AA metabolism and other signaling pathways (Chyb et al., 1999), and this direct activation was thought to be due to the direct ligand-binding of PUFAs to *Drosophila* TRP and TRPL channels (Chyb et al., 1999). Since the
positions of double bond appears to be important for the potency of PUFAs on TRPV3, it is possible that the double-bond at the fifth position from the carboxylated head group promotes the direct binding of PUFAs to TRPV3.
Figure 3.1 AA potentiated TRPV3-mediated Ca²⁺ influx in both mouse keratinocytes and TRPV3-transfected HEK293 cells. Mouse 308 keratinocytes (A) or HEK293 cells expressing TRPV3 (B and C) were loaded with fura2-AM and monitored using Ca²⁺ photometry at room temperature as described in Materials and Methods. Cells were treated with 2APB (300 μM for (A) and 100 μM for (B and C)) and AA (10 or 30 μM as indicated) through constant perfusion for 10 and 20 sec, respectively. Left, representative traces; right, summaries of relative responses for the number of experiments indicated in parentheses. *P<0.05, †P<0.005, **P<0.001, different from control response to 2APB. This experiment was performed by Chunbo Wang.
Figure 3.2 AA specifically potentiated the currents of TRPV3 but not TRPV1 and TRPV2.

A, TRPV3 transient transfected HEK293 cells were repetitively stimulated with 100 µM 2APB. In the later period of 2APB stimulations, 10 µM AA was constantly applied together with 2APB. Cells were held at 0 mV in whole-cell mode, and traces of ramps from -100 mV to +100 mV at 2 Hz were applied to establish IV curves and the time-course of the TRPV3 response. Upper: time course of TRPV3 response at +100 mV (filled circles) and -100 mV (open circles). Note, different scales were used for the outward and inward currents. Lower, I-V curves at the indicated time points.

B, Summary of relative response for TRPV3-transfected cells to 100 µM 2APB in the presence or absence of 10 µM AA. Numbers of experiments were indicated in the parenthesis.

C, Similar to A and B, but TRPV2 was transfected in HEK293 cells. Due to the low efficacy of 2APB to TRPV2, 1 mM 2APB was used to activate TRPV2 currents.

D, Summary of relative response for TRPV2-transfected cells to 1 mM 2APB, n=4

E and F, Similar to (C) and (D) except that TRPV1 was transfected in HEK293 cells and 100 µM 2APB was used, n=6.

This experiment was performed by Hongzhen Hu.
Figure 3.3 Functional characterization of AA-potentiated TRPV3 current.

A and B, Effects of 10 µM ruthenium red (RR) on AA potentiated TRPV3 currents. Representative time course response (A) at -100 mV and +100 mV and I-V curves (B) at indicated time points were shown. AA (10 µM) alone (time point a) did not induce any TRPV3 current, but potentiated 100 µM 2APB evoked TRPV3 current (time point b). RR (10 µM) completely blocked AA potentiated TRPV3 inward current but increased TRPV3 outward current (time point c).

C and D, Summary of inhibitory effect for inward current (D) and facilitatory effect for outward current (C) by RR for TRPV3 currents induced by 2APB alone (open bars, n=6) or 2APB+AA (filled bars, n=8).

E and F, AA dose-dependently potentiated 100 µM 2APB-evoked TRPV3 current. Shown are representative TRPV3 response time course at -100 mV and +100 mV (E) and I-V curves at indicated AA concentrations (F).

G, Dose-response curves of AA potentiation after subtracting the initial currents evoked by 2APB alone. Normalized response at -100 mV and +100 mV were fitted with Hill equation, n=4.

H, AA at 100 µM, the highest concentration of AA used in this study, did not activate TRPV3 current by itself, but strongly potentiated 2APB-induced TRPV3 response. Shown are the time course of TRPV3 response at -100 mV and +100 mV (left) and I-V curves at indicated time points (right).
Figure 3.4 AA increased the open probability of TRPV3.

A, A representative excised inside-out patch held at -100 mV was treated with 10 µM 2APB and 10 µM AA as indicated. Shown are NPo in 2-sec bins (upper), raw current trace (middle), and currents traces at indicated time points in an expanded time scale (lower).

B and C, A representative excised outside-out patch held at -50 mV was treated with 5 µM 2APB and 10 µM AA as indicated. Shown are NPo in 2-sec bins (upper) and raw current trace (lower) for B, and current traces in the indicated time points in an expanded time scale for C.

D and E, Changes in average NPo in individual patches of inside-out (D) and outside-out (E) before (-AA) and after (+AA) the addition of AA.
Figure 3.5 AA potentiated 2APB-evoked TRPV3 currents in *Xenopus* oocytes.

A, TRPV3-injected *Xenopus* oocyte was stimulated with 100 μM 2APB for 3 times as indicated (open bars). AA (10 μM) was included as indicated (black bar) for 1 min in the second stimulation. The oocytes was held at -20 mV and voltage ramps from -100 mV to +100 mV at 1 HZ were used to record TRPV3 currents. Left, time-course of TRPV3 response at +100 mV and -100 mV. Right, I-V curves obtained at indicated time points.

B, Similar to (A) for a different TRPV3-injected oocyte, except 2APB (100 μM) (open bars) was applied for 1.5 min and 10 μM AA (black bars) or 10 μM AEA (gray bars) was applied for 30 sec as indicated.

C, Similar to (B), except TRPV1 instead of TRPV3 was injected. AA and AEA, both at 10 mM, were applied for 30 and 15 sec, respectively.

D, Summary of responses compared to 100 μM 2APB alone at +100 mV (left) and -100 mV (right) for TRPV1 (white bars, n=5) and TRPV3 (gray bars, n=7) injected oocytes.

In this figure, all cRNAs were prepared by Chunbo Wang, and all recordings were performed by Hongzhen Hu.
Figure 3.6 AA dose-dependently potentiated 2APB-evoked TRPV3 currents in Xenopus oocytes.

A, TRPV3-injected oocyte was continuously stimulated with 100 μM 2APB. A gradually increasing concentration of AA (1 μM, 10 μM, 100 μM) was included as indicated. Left, time course of TRPV3 response at -100 mV and +100 mV. Right, I-V curves obtained at indicated time points.

B, Dose-response curves of AA potentiation of TRPV3 in Xenopus oocytes. The initial response to 2APB alone was subtracted for normalization. Curves are fits to the Hill equation for relative responses at both +100 mV (filled circles) and -100 mV (open circles), n=4.

C, Dose-dependence of 2APB-evoked TRPV3 currents in oocytes without (upper) or with 10 mM AA pretreatment (lower). The oocyte was held at -40 mV and continuously recorded at 100 Hz.

D, Dose-response curves of 2APB for TRPV3 currents in the presence (open circles) and absence (filled circles) of 10 μM AA.

In this figure, all cRNAs were prepared by Chunbo Wang, and all recordings were performed by Hongzhen Hu.
Figure 3.7 AA potentiated 2APB-evoked TRPV3 independent of kinases and its downstream metabolites.

A, A representative trace showed that KN-62 at 3 μM had no effect on the potentiation of 2APB-evoked TRPV3 current induced by AA. TRPV3-injected oocyte was held at -40 mV and stimulated with 300 μM 2APB with or without pretreatment of 10 μM AA in the absence or presence of KN-62 as indicated.

B, Summary of effect of kinase inhibitors (KN-62 at 3 μM, Go6979 at 0.3 μM, and Ro318220 at 1 μM) and PLC inhibitor (U73122 at 10 μM) on the AA-potentiated TRPV3 currents. Responses were normalized to those obtained with 2APB+AA.

C and D, Similar to (A) and (B) except AA metabolic blockers were used as indicated. Except piroxicam at 60 μM, all blockers were tested at 10 μM.

E, Representative traces of the direct effects of some AA metabolic blockers on 300 μM 2APB-evoked TRPV3 currents. All blockers were tested at 10 μM.

F, Summary of responses normalized to that obtained with 2APB alone. *P<0.01 different from the response with 2APB alone, and the numbers of experiment were indicated in the parenthesis.

G, Structures of 4 AA metabolic blockers that had direct potentiation effect on 2APB-evoked TRPV3 current.

In this figure, all cRNAs were prepared by Chunbo Wang, and all recordings were performed by Hongzhen Hu.
Figure 3.8 Multiple unsaturated fatty acids potentiated 2APB evoked TRPV3 currents in *Xenopus* oocytes.

A, TRPV3-injected oocytes were stimulated with 300 μM 2APB in the presence or absence of 10 μM various free unsaturated fatty acids. Responses were normalized to that obtained with 2APB plus 10 μM AA, and dashed line indicated the response level to 2APB alone.

B, Structures of selected fatty acids which displayed relatively strong potentiation effect on 2APB-evoked TRPV3 currents.

In this figure, all cRNAs were prepared by Chunbo Wang, and all recordings were performed by Hongzhen Hu.
CHAPTER 4

CALCIUM PLAYS A CENTRAL ROLE IN THE SENSITIZATION OF TRPV3

The main discovery presented in this chapter has been published in 2008 (Xiao et al., 2008a). Most experiments were performed by Rui Xiao except that figure 4.5 was provided by Dr. Jisen Tang. This represents the nature of the collaboratory research in our laboratory.

4.1 Introduction

TRPV3 is widely distributed in various tissue types. In addition for central and peripheral nerve systems, it is also expressed in many other non-neuronal cell types, including skin, stomach, tongue, hair follicles, adipose, and placenta (Smith et al., 2002; Peier et al., 2002b; Xu et al., 2002; Xu et al., 2006; Asakawa et al., 2006), indicating that TRPV3 may play functions unrelated to sensory in those non-neuronal cells. In mouse, unlike TRPV1 and TRPV2, TRPV3 is not expressed in DRG neurons, but in contrast, highly expressed in skin keratinocytes (Peier et al., 2002b) and tongue epithelium (Xu et al., 2006). Studies with TRPV3 knockout mice have shown that TRPV3 deficient mice displayed impaired hot temperature sensing in the noxious and innocuous ranges;
therefore, TRPV3 plays a role in sensing environmental hot temperature (Moqrich et al., 2005). The first synthetic chemical agonist for TRPV3, 2APB, was found by our laboratory and others (Hu et al., 2004; Chung et al., 2004). Later, several other naturally occurring compounds from plants were also found to activate TRPV3, including camphor (Moqrich et al., 2005), carvacrol, eugenol and thymol (Xu et al., 2006). These naturally occurring compounds are widely used in medicines, food sensitizers, and some of them are known to be allergens (Moqrich et al., 2005; Xu et al., 2006); it is possible that TRPV3 channels in skin and tongue are the primary target for these compounds.

Interestingly, when first cloned, the response of TRPV3 was shown to be sensitized upon repetitive heat stimulation by two independent groups (Peier et al., 2002b; Xu et al., 2002). This phenomenon was confirmed by subsequent studies in other laboratories in both expression systems and native keratinocytes (Chung et al., 2004; Hu et al., 2004). Moreover, the sensitization of TRPV3 is independent of stimuli source: all known TRPV3 activators are able to induce TRPV3 sensitization by repetitive stimulations; and distinct stimuli cross-sensitize TRPV3 as well. For instance, camphor and carvacrol sensitizes the response of TRPV3 to heat (Moqrich et al., 2005; Xu et al., 2006) and DPBA sensitizes the response of TRPV3 to engenol (Xu et al., 2006). Among the TRP channel superfamily, TRPV3 is the only member that consistently shows sensitization upon stimulations, and sensitization property of TRPV3 is also somewhat unique among all membrane proteins including ion channels and receptors which
normally display desensitization upon repetitive or prolonged stimulations. Given that TRPV3 sensitization is independent of stimulus type, it appears that sensitization is an intrinsic property of the TRPV3 channel.

4.2 Intracellular Ca\textsuperscript{2+} buffering strength affects the sensitization kinetics of TRPV3

TRPV3 is a Ca\textsuperscript{2+} permeable cation channel with a permeability ratio of \(\text{Ca}^{2+} (P_{Ca})\) to Na\textsuperscript{+} (\(P_{Na}\)) \(P_{Ca}/P_{Na}\) at about ten (Xu et al., 2002). Since many Ca\textsuperscript{2+} permeable ion channels are under the regulation by Ca\textsuperscript{2+}, we examined the role of intracellular Ca\textsuperscript{2+} on the regulation of TRPV3. A convenient way to control intracellular Ca\textsuperscript{2+} concentrations is to use whole-cell patch clamping technique to infuse cells with Ca\textsuperscript{2+} of defined concentrations in intracellular solutions. Two Ca\textsuperscript{2+} chelators are normally used to clamp intracellular [Ca\textsuperscript{2+}]: EGTA and BAPTA. EGTA and BAPTA have similar affinity to calcium ions, but BAPTA binds to Ca\textsuperscript{2+} about 50-400 fold faster than EGTA (Tsien, 1980). We used 2APB as the agonist to activate TRPV3 current, by comparing TRPV3 currents of cells with different Ca\textsuperscript{2+} buffering capabilities, we should be able to detect any putative regulation of TRPV3 by intracellular Ca\textsuperscript{2+}. Two approaches were tried at the initial test by either continuously stimulating or repetitively stimulating TRPV3-expressing HEK293 cells with 2APB.

Indeed, intracellular Ca\textsuperscript{2+} clearly plays a role in the sensitization kinetics of TRPV3. During the same period of continuous stimulation (10 min) by 100 μM
2APB, TRPV3 current developed much faster and reached much higher amplitudes when intracellular Ca\(^{2+}\) was buffered by 10 mM BAPTA than by 1 mM EGTA (Fig 4.1 A and B). The plateau phase of TRPV3 current was typically achieved after about 6 min stimulation when intracellular solution contained 10 mM BAPTA, but it was not reached for cells with intracellular solution containing 1 mM EGTA even at the end of the stimulation. During the continuous application of 2APB, switching regular ECS to the N-methyl-D-glucamine (NMDG\(^+\)) solution in which all cations were replaced with NMDG\(^+\) completely blocked TRPV3 inward current in both 10 mM BAPTA and 1 mM EGTA buffered cells (Fig 4.1 A and B). This is expected because NMDG\(^+\) is a much larger cation and not permeable to almost all major cation channels; thus no NMDG\(^+\) influx (inward current) for TRPV3 was anticipated. However, an interesting phenomenon came from the outward current of TRPV3. Surprisingly, the outward current of TRPV3 was also largely blocked by NMDG\(^+\) under both conditions (Fig 4.1 A and B), whereas for many other cation channels, the outward current was typically not affected by NMDG\(^+\). Given that TRPV3 has a particularly large single channel conductance of about 170 pS (Xu et al., 2002), it is possible that the pore size of TRPV3 is large enough to allow NMDG cation to plug-in and block the pore, thus inhibiting both inward and outward TRPV3 currents.

Sensitization process was also observed for TRPV3 by repetitive 2APB stimulations with short intervals (Fig4.2 A and B). Again, the 2APB-evoked TRPV3 current developed much faster and typically reached plateau phase after
about six stimulations when intracellular Ca\(^{2+}\) was buffered with 10 mM BAPTA (Fig 4.2 A). For cells with intracellular Ca\(^{2+}\) buffered by 1 mM EGTA, the 2APB-evoked current gradually increased and finally reached a plateau phase after about 18 stimulations (Fig 4.2 B). As a control, we also stimulated TRPV3-expressing HEK293 cells twice with a 10 min interval (same as the total stimulation time for Fig 4.2 A and B), but no sensitization occurred under this condition (Fig 4.2 C), suggesting that the sensitization process was not due to the loss of intracellular contents or other intracellular changes during the long period of whole-cell dialysis, and sensitization property is intrinsic for TRPV3.

Using an on-line program called “Pore”, developed by J. Kenton, we calculated the relationship between free [Ca\(^{2+}\)], and the distance from the pore. Assuming the free local [Ca\(^{2+}\)], at the exit site of the channel pore to be 1 mM, the diffusion coefficient for Ca\(^{2+}\) to be 0.22 \(\mu\)m\(^2\)/ms, dissociation constants for Ca\(^{2+}\)/EGTA to be 1.8 \(\times\) 10\(^{-7}\) fmol/\(\mu\)m\(^3\) and Ca\(^{2+}\)/BAPTA to be 2.2 \(\times\) 10\(^{-7}\) fmol/\(\mu\)m\(^3\), binding rate of Ca\(^{2+}\) to EGTA to be 2.5 \(\times\) 10\(^3\) \(\mu\)m\(^3\)/fmol•ms and Ca\(^{2+}\) to BAPTA to be 4 \(\times\) 10\(^5\) \(\mu\)m\(^3\)/fmol•ms (Naraghi & Neher, 1997), three Ca\(^{2+}\)-buffering solutions were compared. For 10 mM BAPTA buffered solution, within 10 nm from the pore, free [Ca\(^{2+}\)] should drop to less than 1 nM, whereas for 10 mM EGTA or 1 mM EGTA buffered solution, it takes up to 33 nm from the pore to decrease the free [Ca\(^{2+}\)] to lower than 1 nM (Fig 4.2 E). The pore region of the first crystallized K\(^+\) channel, KcsA, was 34 Å in length (Doyle et al., 1998). Considering that first, KcsA is a prokaryotic K\(^+\) channel and has a much smaller molecular weight than eukaryotic
K⁺ channels; second, mammalian TRP channels are typically at least 2-3 fold larger than mammalian voltage-gated K⁺ channels; and third, TRPV3 channel has relatively long and flexible C and N-termini, we can reasonably estimate that the overall size of a single TRPV3 molecule was in 1-10 nm diameter range. Therefore, the difference in distance between BAPTA and EGTA solutions to allow Ca²⁺ to travel might be critical for local Ca²⁺ regulation of ion channels. From the simulation, we found that 10 mM EGTA and 1 mM EGTA were very similar in regard to the distance free Ca²⁺ can travel within the cell. Therefore, we predicted that the sensitization kinetics of TRPV3 in 1 mM EGTA buffered- or 10 mM EGTA buffered-TRPV3 expressing cells should be similar. Indeed, we found no obvious difference for TRPV3 sensitization in the two buffered intracellular solutions with 1 and 10 mM EGTA(Fig 4.2 D).

The opening of TRPV3 channel induces Ca²⁺ influx through the pore. The local Ca²⁺ close to the pore reaches higher concentration and diffuses further when EGTA is used for buffering. If the different sensitization kinetics observed between EGTA and BAPTA buffered intracellular solution was due to the local Ca²⁺ dynamics, slower sensitization in EGTA-buffered pipette solution indicates that intracellular Ca²⁺ plays an inhibitory effect on the activation of TRPV3. If this is the case, Ca²⁺ influx through fully sensitized TRPV3 channels (which means the same [Ca²⁺] at the starting point) should display different rate of channel inactivation in EGTA- and BAPTA-buffered cells. Indeed, we found that the inactivation of TRPV3 inward current (which carries Ca²⁺ influx) was more
predominant when intracellular solution was buffered by 1 mM EGTA than by 10 mM BAPTA (Fig 4.2 F).

4.3 Functional characterization of TRPV3 sensitization

For TRPV1 and TRPM8, a gradual shift of voltage-dependence was thought to be the underlying mechanism for cold and chemical sensitivities of these TRP channels (Voets et al., 2004). To further characterize the sensitization process of TRPV3 under two distinct Ca\(^{2+}\) buffering strengths, we performed a series of detailed whole-cell recordings to study if and how voltage-dependence of TRPV3 was changed during sensitization. Two protocols were used alternatively: a step protocol was performed to measure tail currents which reflex the relative open probability of the channel and allow us to establish conductance-voltage (GV) relationship, and a re-ramp protocol was performed to monitor the sensitization process. To establish the G-V curves of TRPV3, tail currents at -100 mV immediately following voltage steps (300 ms) to different potentials from -100 to +200 mV with a 20-mV increment were recorded to represent relative conductance (G). A modified Boltzmann equation was used to fit the G-V curves:

\[
\frac{I_{\text{tail}}}{I_{\text{max}}} (%) = b + a(1 + \exp(-(V - V_{1/2})/s)),
\]

where \(V_{1/2}\) is the potential for half-maximal channel activation, \(b\) is the voltage-independent fraction of the total activity, \(a\) is the voltage-dependent fraction (VDF), and \(s\) is the slope factor which equals \(RT/zF\), with \(z\) being the valence of the gating charge.
Consistently, the sensitization process developed much faster when 10 mM BAPTA was used in the intracellular solution compared to that with 1 mM EGTA (Fig 4.3 A and B). During the sensitization process induced by repetitive 2APB stimulations, a gradual left-shift of the G-V curve was observed for both EGTA- and BAPTA-buffered TRPV3-expressing cells (Fig 4.3 C). However, the shift was faster for BAPTA-buffered cells where in the tenth stimulation it already stabilized; whereas it took about 17 stimulations for the G-V curve to stabilize for EGTA-buffered cells (Fig 4.3 C). The $V_{1/2}$ value for 2APB-evoked TRPV3 activation decreased from $+144 \pm 12$ mV (the first 2APB stimulation, naïve state) to $+47 \pm 11$ mV (the 24th stimulation, sensitized state) when intracellular Ca$^{2+}$ was buffered by 1 mM EGTA, whereas it changed from $77 \pm 14$ mV (first stimulation) to $13 \pm 4$ mV (the 24th 2APB stimulation, sensitized state) when intracellular Ca$^{2+}$ was buffered by 10 mM BAPTA (Fig 4.3 D, upper panel).

Interestingly, the $V_{1/2}$ values for naïve state cells with EGTA intracellular solution and BAPTA intracellular solution are significantly different (Fig 4.3 D, upper panel) and the TRPV3 current amplitude for the first 2APB stimulation was much larger with BAPTA intracellular solution than with EGTA intracellular solution (Fig 4.2 A and B). This indicates that even for the non-stimulated cells, TRPV3 is still under intrinsic inhibition by intracellular basal Ca$^{2+}$, which is removed to a larger extent when 10 mM BAPTA was infused into the cells. Pre-existing inhibition by intracellular basal Ca$^{2+}$ through CaM also occurs for many other cation channels, such as CNG channels (Bradley et al., 2004) and voltage-gated Ca$^{2+}$ channels.
The valence of gating charge, $z$, was not changed during sensitization under both conditions (Fig 4.3 D middle panel), implying that the numbers of charges that have to move in order for the channel to open is not affected by intracellular Ca$^{2+}$ buffering. Moreover, we also observed a slight increase in the voltage-independent fraction of TRPV3 activity during sensitization, especially when intracellular Ca$^{2+}$ was buffered by 10 mM BAPTA (Fig 4.3 D lower panel).

4.4 Intracellular Ca$^{2+}$ inhibits TRPV3 through CaM

CaM is the most important intracellular Ca$^{2+}$ sensor and ubiquitously expressed in all eukaryotic cell types (Crivici & Ikura, 1995). As discussed in the introduction part of this thesis, many if not all TRP channels are regulated by CaM. Since intracellular Ca$^{2+}$ appeared to inhibit TRPV3, we reasoned that CaM might play a role for this Ca$^{2+}$-induced inhibition. We tested the effect of several established CaM antagonists, including ophiobolin A, W7, and calmidazolium, on the sensitization process of TRPV3.

In order to block CaM function effectively, most CaM antagonists require some free Ca$^{2+}$. Therefore, we added some Ca$^{2+}$ into 10 mM BAPTA buffered intracellular solution to clamp the free [Ca$^{2+}$] at 1.6 μM. Consistent with our expectation, increasing free [Ca$^{2+}$] in 10 mM BAPTA-buffered intracellular solution significantly slowed down the sensitization process of TRPV3 and transform it to be more like that with 1 mM EGTA-buffered intracellular solution.
(Fig 4.4 A, compared with Fig 4.2 A and B). However, by adding 20 μM ophiobolin A into the same intracellular solution used for Fig 4.4 A, the sensitization process of TRPV3 was largely accelerated and the initial response to 2APB was significantly increased (Fig 4.4 B and C). In fact, after about 8 times of 2APB stimulation, TRPV3 current reached the maximum and then even started desensitization (Fig 4.4 B). We also obtained similar results using 100 μM W7 and 10 μM calmidazolium (data not shown). Therefore, intracellular CaM indeed inhibits TRPV3. It appears that an intrinsic inhibition for TRPV3 pre-exists before any stimulation (which was demonstrated by the different TRPV3 current sizes between 1 mM EGTA- and 10 mM BAPTA-buffered intracellular solution), it is possible that Ca-CaM permanently binds to TRPV3 channel under resting conditions (which is the case for CNG channel (Bradley et al., 2004)) and underlies the intrinsic inhibition of TRPV3.

4.5 Identification of a CaM-binding site in the N-terminus of TRPV3

In order to identify any putative CaM-binding site on TRPV3, a series of TRPV3 fragments were synthesized and labeled with [35S]methionine, and then tested for binding to CaM-agarose in the absence or presence of 50 μM Ca²⁺. Multiple putative CaM-binding sites in both N- and C-termini were identified by the in vitro binding assay. A fragment from the N-terminus (fragment I corresponding to aa 108-130) of TRPV3 showed the strongest binding to CaM (Fig 4.5 A and B). CaM is a negatively charged molecule (Clapham, 2008); therefore, the critical amino acids that directly interact with CaM are normally positively charged. We mutated
all positively charged amino acids within this region to neutral or negatively charged ones (113RQKKKRLKKR122 to SQAEASDAEG). Consistent with our expectation, after mutation, this fragment could no longer bind to CaM (Fig 4.5 C). In order to test the effect of this CaM-binding deficient mutant on TRPV3 whole-cell current, we introduced the mutation into full-length wild type TRPV3 and designated the mutant as TRPV3RK⁻.

4.6 Functional study of TRPV3RK⁻
TRPV3RK⁻ was successfully expressed in HEK293 cells. Strikingly, we found that the 2APB-evoked currents for TRPV3RK⁻ with 1mM EGTA-buffered intracellular solution were very similar to those for wild type TRPV3 with 10 mM BAPTA-buffered intracellular solution, i.e., very fast sensitization process and large response to the first 2APB stimulation (Fig4.6 A, compared with Fig4.3 B). Using similar analysis for Fig 4.3, we established the G-V curves of TRPV3RK⁻ mutant with both 1 mM EGTA- and 10 mM BAPTA-buffered intracellular solutions. A small left shift in the G-V curves was observed with both solutions after repetitive challenges with 2APB. However, the changes in $V_{1/2}$ and $z$ values are not statistically significant (Fig 4.6 B and C). Moreover, the 2APB-evoked currents at both naïve-state (1ˢᵗ stimulation) and the sensitized state (23ʳᵈ stimulation) were not different for 1 mM EGTA or 10 mM BAPTA buffered TRPV3RK⁻-expressing cells in terms of current densities (Fig 4.6 D).
From the studies with the CaM-binding deficient mutant TRPV3RK− we found that the pre-existing inhibition of TRPV3 was largely abolished (the initial response to 2APB stimulation was already comparable to the maximal response). Therefore, it supports the idea that CaM binding to the N-terminal site of TRPV3 inhibits the channel. When 10 mM BAPTA but not 1 mM EGTA was used to tightly control local Ca2+ fluctuation, somehow the binding of the pre-existing CaM to TRPV3 was lost or weakened and the channel was released from the inhibition by CaM, displaying larger initial response and faster sensitization. This entire process was mimicked by the TRPV3RK− mutant that loses the important binding site to CaM regardless whether intracellular Ca2+ was buffered by 1 mM EGTA or 10 mM BAPTA. However, in our in vitro assay, several other regions of TRPV3 also display weaker CaM binding affinities, so we cannot rule out the possibility that CaM still regulates TRPV3 through other binding sites.

4.7 TRPV3 is also inhibited by extracellular Ca2+

If intracellular Ca2+/CaM inhibits TRPV3, by stimulating TRPV3 with 2APB in the absence of extracellular Ca2+, Ca2+ influx through the opened TRPV3 channel would be avoided. Theoretically, no [Ca2+]i increase should be expected and intracellular Ca2+ inhibition of TRPV3 should be largely relieved. We stimulated TRPV3-expressing cells with a constant concentration of 100 μM 2APB but switched the extracellular solution (ECS) between the one that contained 2 mM Ca2+ and the one that was Ca2+-free (omitting Ca2+ and adding 0.1 mM EGTA). An instantaneous current increase was observed when extracellular Ca2+ was
removed, and the increase was reversed without any delay after reintroduction of extracellular Ca$^{2+}$ (Fig 4.7 A). Notably, the I-V curve for the 2APB-stimulated currents in the absence of extracellular Ca$^{2+}$ became linear instead of double-rectifying in the presence of extracellular Ca$^{2+}$. Therefore, it appears that similar to intracellular Ca$^{2+}$, extracellular Ca$^{2+}$ also inhibits TRPV3 channel but in a more instantaneous manner.

The next question we asked was whether the sensitization of TRPV3 to repetitive stimulation was changed in the absence of extracellular Ca$^{2+}$. We did similar experiments as Fig 4.2 A and B, but omitting extracellular Ca$^{2+}$. Consistent with our expectation, the sensitization process of TRPV3 in the absence of extracellular Ca$^{2+}$ was accelerated. When intracellular solution was buffered by 10 mM BAPTA, there was almost no sensitization and the initial response to 100 μM 2APB was similar to the maximum (Fig 4.7 B); when intracellular solution was buffered by 1 mM EGTA, although sensitization to repetitive stimulation was still obvious, it had a faster kinetics (it took about 12 stimulations to reach the maximum whereas 18 stimulations were required when extracellular Ca$^{2+}$ was present) (compare Fig 4.7 C and Fig 4.2 B). Importantly, TRPV3 currents induced by 100 μM 2APB were always much larger under both intracellular buffering conditions by omitting extracellular Ca$^{2+}$ (compare Fig 4.7 D and Fig 4.2 D), supporting again the argument that extracellular Ca$^{2+}$ inhibits TRPV3.
By removing extracellular Ca\textsuperscript{2+}, the inhibition of TRPV3 due to Ca\textsuperscript{2+} influx should be abolished. However, the sensitization of TRPV3 was still present especially when intracellular solution was buffered by 1 mM EGTA (Fig 4.7 C and summary data D), therefore, the intrinsic intracellular inhibition of TRPV3 was not dependent on Ca\textsuperscript{2+} influx \textit{per se}, but when extracellular Ca\textsuperscript{2+} was present and entered cytosol, it added a further inhibition to TRPV3. It is likely that the intrinsic intracellular inhibition of TRPV3 is due to the pre-bound Ca\textsuperscript{2+}/CaM to the channel. During prolonged or repetitive agonist stimulation, the conformation of the channel gradually changes and somehow the binding affinity of the N-terminal CaM-binding site to Ca\textsuperscript{2+}/CaM gradually decreases and eventually it loses the CaM binding regardless the cytosolic [Ca\textsuperscript{2+}], giving rise to a fully sensitized TRPV3. When Ca\textsuperscript{2+} is present extracellularly, Ca\textsuperscript{2+} binds to CaM and slows down the departure of CaM from TRPV3. Therefore, the most critical component for the sensitization phenomenon is intracellular CaM. In order to prove that the sensitization observed in the absence of extracellular Ca\textsuperscript{2+} was also due to intracellular CaM inhibition, we tested the effect of CaM antagonist ophiobolin A on 2APB-evoked TRPV3 current in the absence of extracellular Ca\textsuperscript{2+}. Consistent with the phenomenon in Fig 4.4, when extra Ca\textsuperscript{2+} was added into 10 mM BAPTA-buffered solution to make a 1.6 \textmu M free Ca\textsuperscript{2+}-clamped intracellular solution, sensitization of TRPV3 was obvious even in the absence of extracellular Ca\textsuperscript{2+}, and 20 \textmu M ophiobolin almost completely abolished the sensitization process.
Very interestingly, we found that even in the absence of extracellular Ca\(^{2+}\), 100 μM 2APB treatment of TRPV3-expressing HEK293 cells still induced a [Ca\(^{2+}\)]\(_i\) increase, which was smaller than when extracellular Ca\(^{2+}\) was present (Fig 4.7 F). Therefore, only the difference of [Ca\(^{2+}\)]\(_i\) increase between cells measured with extracellular Ca\(^{2+}\) and those without extracellular Ca\(^{2+}\) was due to the Ca\(^{2+}\) influx mediated by the opening of TRPV3 channels on plasma membrane. The residual [Ca\(^{2+}\)]\(_i\) increase observed in the absence of extracellular Ca\(^{2+}\) was due to Ca\(^{2+}\) release from 2APB sensitive Ca\(^{2+}\) stores. Many if not all TRP channels are also expressed intracellularly and some intracellular TRP channels form functional Ca\(^{2+}\) release channels in intracellular organelles, such as TRPV1 (Liu et al., 2003). TRPV3 has been shown to mainly localize intracellularly in skin keratinocytes (Chung et al., 2004). Since 2APB is a membrane permeable TRPV3 agonist, it is likely that 2APB can activate TRPV3 located on intracellular membranes and induce Ca\(^{2+}\) release. If this is true, Ca\(^{2+}\) release from 2APB sensitive internal stores should be the main Ca\(^{2+}\) source involved in the initial inhibition of TRPV3 when cells were recorded using the extracellular Ca\(^{2+}\)-free solution.

4.8 Characterization of extracellular Ca\(^{2+}\) block of TRPV3

Since extracellular Ca\(^{2+}\) also inhibits TRPV3, we studied the dose-dependence of extracellular Ca\(^{2+}\) on the inhibition of TRPV3. TRPV3-expressing cells were constantly stimulated with 100 μM 2APB for six times, while the free Ca\(^{2+}\) concentration was gradually increased from virtually no Ca\(^{2+}\) to 30 mM. A step-
wise inhibition was observed when extracellular free Ca\(^{2+}\) concentration was increased (Fig 4.8 A). Interestingly, the initial inhibition of TRPV3 current due to the switch from <10 nM [Ca\(^{2+}\)] solution to 3 \(\mu\)M [Ca\(^{2+}\)] solution was much larger for the first than for the sixth 2APB stimulation. For the first stimulation, the initial block was more than 50% while for the sixth the block was only about 10% (Fig 4.8 A, also see the I-V curve of Fig 4.8 B). The relationship between extracellular free Ca\(^{2+}\) concentration and TRPV3 current was best fitted with a two-site formula: 

\[
\frac{I_{[Ca]}}{I_{\text{max}}} = \frac{f_H}{(1 + [Ca]/k_H)} + \frac{(1 - f_H)}{(1 + [Ca]/k_L)},
\]

where \(f_H\) is the fraction of the high affinity state, and \(k_H\) and \(k_L\) are IC\(_{50}\) values of the high and low affinity state, respectively (Fig 4.8 C). Dataset from Fig 4.8 C indicates that two extracellular Ca\(^{2+}\)-binding sites might exist for TRPV3, with the high affinity in low micromolar range while the low affinity in millimolar range. Interestingly, a gradual right-shift of the extracellular Ca\(^{2+}\) dose-response curve was observed (Fig 4.8 C), indicating that the inhibition by extracellular Ca\(^{2+}\) was gradually relieved, but not completely released, during sensitization of TRPV3. The IC\(_{50}\) values for the high affinity Ca\(^{2+}\)-binding site increased from 1.42 ± 0.26 and 1.50 ± 0.21 \(\mu\)M to 9.0 ± 1.2, and 8.0 ± 1.1 \(\mu\)M at -100 and +100 mV, respectively (Fig 4.8 D).

4.9 Identification of a high-affinity Ca\(^{2+}\)-binding site in the putative pore region of TRPV3

The instantaneous effect of extracellular Ca\(^{2+}\) (Fig 4.7 A) suggests that Ca\(^{2+}\) may directly bind to a negatively-charged amino acid within the TRPV3 pore and
blocks the channel. Therefore, in a systematic screening of all negatively charged amino acids in the putative pore region of TRPV3, we identified a critical site Asp641 involved in the extracellular Ca\(^{2+}\) block of TRPV3. After neutralizing this Asp641 to Asn (TRPV3D641N), we no longer observed the initial block of TRPV3 current when extracellular [Ca\(^{2+}\)] was increased from <10 nM to 3 \(\mu\)M, indicating that the inhibition due to the high affinity Ca\(^{2+}\)-binding was abolished. However, the inhibition by high [Ca\(^{2+}\)] still existed, indicating that the low-affinity Ca\(^{2+}\)-binding site was still intact (Fig 4.9 A). The data for the inhibition dose-response effect of extracellular Ca\(^{2+}\) was best fitted by the standard Hill equation:

\[
l_{\text{IC50}}/l_{\text{max}} = [\text{Ca}^{2+}]^{h}/(K^{h}+[\text{Ca}^{2+}]^{h})\]

where \(K\) is the apparent dissociation constant and \(h\) is the hill coefficient. The IC\(_{50}\) values for TRPV3D641N were 1.12 ± 0.25 and 3.21 ± 0.32 \(\mu\)M for -100 and +100 mV, respectively (Fig 4.9 B).

4.10 Double mutations largely abolished Ca\(^{2+}\) inhibition of TRPV3

Consistent with our hypothesis that intracellular Ca\(^{2+}\)/CaM was the most critical component for the sensitization property of TRPV3, D641N mutant of TRPV3 still displayed sensitization process especially when intracellular Ca\(^{2+}\) was buffered by 1 mM EGTA, and the overall sensitization process was still much faster when intracellular Ca\(^{2+}\) was buffered by 10 mM BAPTA than that buffered by 1 mM EGTA (Fig 4.10 A and B). The TRPV3D641N mutation abolished the high affinity Ca\(^{2+}\) block. In general, this mutant displayed a much larger current than wild type TRPV3 (Fig 4.10 A and B, comparing with Fig 4.2 A and B).
More Ca$^{2+}$ influx was expected with the TRPV3D641N mutant because of the much larger current size. Therefore, in theory the inhibition of TRPV3 due to Ca$^{2+}$ influx should be more dramatic for the D641N mutant and the sensitization process of the mutant should also be slower because of the additional inhibition induced by Ca$^{2+}$ influx. Surprisingly, the sensitization process for TRPV3D641N was even faster than wild type TRPV3. Two possibilities could explain this phenomenon. First, the D641N mutation in the putative pore region of TRPV3 somehow changes the permeability ratio of Ca$^{2+}$ to Na$^+$. Instead of increasing Ca$^{2+}$ influx, less Ca$^{2+}$ is permeable by the mutant. Second, although the N-terminal CaM-binding site is the most important for the sensitization property of TRPV3, the pore region of TRPV3 is also involved. A channel pore diameter and ion selectivity change due to stimulation strength has been found for TRPV1 (Chung et al., 2008). It is possible that TRPV3 pore has a similar property and during sensitization, the pore size of TRPV3 gradually increases, giving rise to increased current size. However, both hypotheses require further investigation.

We combined the two mutations of TRPV3 in order to remove both intracellular Ca$^{2+}$/CaM inhibition and the extracellular Ca$^{2+}$ block (the double mutant was designated as TRPV3D641N-RK$^-\lambda$). Consistent with our expectation, the response of double mutant to 100 μM 2APB was almost the same for both 1mM EGTA buffered or 10 mM BAPTA buffered cells (Fig 4.10 G). Compared with TRPV3D641N mutant, the double mutant showed larger current density and faster activation kinetics because of the abolishment of the intracellular CaM
binding (Fig 4.10 C to F). Taken together, our results with the double mutant TRPV3 D641N-RK demonstrate again that TRPV3 is inhibited by both intracellular and extracellular Ca$^{2+}$.

4.11 Summary

TRPV3 is unique in the TRP superfamily in that it displays sensitization upon repetitive or prolonged stimulation. High expression level of TRPV3 has been confirmed in skin and epithelia of tongue and nose. Given that TRPV3 is sensitive to multiple skin sensitizers, flavors and irritants commonly used in daily lives, including camphor, carvacrol, ethyl vanillin, and eugenol, it is possible that TRPV3 is the primary target of these compounds and responsible for chemesthetic sensations induced by them. Due to the unique sensitization property of TRPV3, it is highly likely that TRPV3 sensitization underlies the molecular mechanism of enhanced oral and nasal sensitivities to the successive exposures of certain odors, flavors, and irritants. Therefore, it is important to understand the mechanism of TRPV3 sensitization.

Our experiments show that Ca$^{2+}$ inhibits TRPV3 from both extracellular site and intracellular sites. We have identified the primary action sites of Ca$^{2+}$ for TRPV3: aa108-aa130 is the major CaM-binding site responsible for the intracellular Ca$^{2+}$/CaM inhibition, and Asp641 is the high-affinity binding site for extracellular Ca$^{2+}$ and responsible for the extracellular Ca$^{2+}$ block. Intracellular CaM-binding site might be more important for the sensitization property of TRPV3 because
TRPV3RK showed large initial response and virtually no sensitization, whereas Asp641 in the putative pore region is important for the direct extracellular Ca\(^{2+}\) binding. Importantly, this aspartic acid is conserved in the TRPV subfamily. The counterpart sites in rat TRPV1 and mouse TRPV4 are Asp646 and Asp682, respectively. Both sites in TRPV1 and TRPV4 have been shown to directly bind to divalent cations and neutralization of these sites have been shown to dramatically change the ruthenium red sensitivity, divalent cation permeability, and Ca\(^{2+}\) inhibition (Chung et al., 2005; Voets et al., 2002). Our data strongly support that Asp641 in TRPV3 plays a similar role in directly binding to extracellular Ca\(^{2+}\), which in turn blocks the channel.

Ca\(^{2+}\) inhibits TRPV3 from both intracellular and extracellular sites. A gradual release of Ca\(^{2+}\) inhibition underlies the sensitization phenomenon of TRPV3. However, what causes a gradual decrease of Ca\(^{2+}\) inhibition to TRPV3 is still unknown. Several possibilities could exist. First, during repetitive or prolonged stimulation, the overall conformation of TRPV3 channel is changed and the N-terminus of TRPV3 departed from the pore region so that local available [Ca\(^{2+}\)] significantly decreases. In addition, the binding affinity of TRPV3 to intracellular Ca\(^{2+}/CaM\) decreases, releasing the channel from intracellular inhibition and displaying sensitization. Second, other intracellular factors might also regulate this process. For example, PUFAs in part 3 of this thesis have been shown to strongly potentiate the TRPV3 channel. It is possible that during repetitive or
prolonged stimulation, the concentrations of PUFAs are gradually increased, which modulates the binding of TRPV3 to CaM and potentiates the channel.
Table 4.1 Solutions used for whole-cell recording experiments.
All concentrations are in mM. Osmolarities of all solutions range from 288 to
305 mOsm/kg. For Ca^{2+}-buffered external solutions, total Ca^{2+} concentrations
were determined using the MaxChelator program
(http://www.stanford.edu/~cpatton/maxc.html).

<table>
<thead>
<tr>
<th></th>
<th>Monovalent</th>
<th>Divalent</th>
<th>Ca^{2+} buffer</th>
<th>Glucose buffer</th>
<th>pH value</th>
<th>adjusted by</th>
</tr>
</thead>
<tbody>
<tr>
<td>Internal solutions:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 EGTA</td>
<td>140 CsCl</td>
<td>0.6 MgCl₂</td>
<td>1 Cs-EGTA</td>
<td></td>
<td>10 Heps</td>
<td>7.2</td>
</tr>
<tr>
<td>10 EGTA</td>
<td>108 CsCl</td>
<td>0.6 MgCl₂</td>
<td>10 Cs-EGTA</td>
<td></td>
<td>10 Heps</td>
<td>7.2</td>
</tr>
<tr>
<td>1 BAPTA</td>
<td>140 CsCl</td>
<td>0.3 Mg-ATP</td>
<td>1 K-BAPTA</td>
<td></td>
<td>10 Heps</td>
<td>7.2</td>
</tr>
<tr>
<td>10 BAPTA</td>
<td>108 CsCl</td>
<td>0.6 MgCl₂</td>
<td>10 Cs-BAPTA</td>
<td></td>
<td>10 Heps</td>
<td>7.2</td>
</tr>
<tr>
<td>1.6 μM Ca^{2+}</td>
<td>90 CsCl</td>
<td>8.87 CaCl₂/0.6 MgCl₂</td>
<td>10 Cs-BAPTA</td>
<td></td>
<td>10 Heps</td>
<td>7.2</td>
</tr>
<tr>
<td>External solutions:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>145 NaCl</td>
<td>2 CaCl₂/1 MgCl₂</td>
<td></td>
<td>10 Heps</td>
<td>10 Heps</td>
<td>7.4</td>
</tr>
<tr>
<td>NMDG</td>
<td>150 NMDG-Cl</td>
<td></td>
<td></td>
<td>10 Heps</td>
<td>10 Heps</td>
<td>7.4</td>
</tr>
<tr>
<td>Ca^{2+}-free</td>
<td>145 NaCl</td>
<td>1 MgCl₂</td>
<td>0.1 Na-EGTA</td>
<td>10 Heps</td>
<td>10 Heps</td>
<td>7.4</td>
</tr>
<tr>
<td>pCa ≥8</td>
<td>145 NaCl</td>
<td>1 MgCl₂</td>
<td>1 Na-EGTA</td>
<td>10 Heps</td>
<td>10 Heps</td>
<td>7.4</td>
</tr>
<tr>
<td>10⁻⁴ Ca^{2+}</td>
<td>145 NaCl</td>
<td>0.59 CaCl₂/1 MgCl₂</td>
<td>1 Na-EGTA</td>
<td>10 Heps</td>
<td>10 Heps</td>
<td>7.4</td>
</tr>
<tr>
<td>3x10⁻⁴ Ca^{2+}</td>
<td>145 NaCl</td>
<td>0.81 CaCl₂/1 MgCl₂</td>
<td>1 Na-EGTA</td>
<td>10 Heps</td>
<td>10 Heps</td>
<td>7.4</td>
</tr>
<tr>
<td>0.001 Ca^{2+}</td>
<td>145 NaCl</td>
<td>0.94 CaCl₂/1 MgCl₂</td>
<td>1 Na-EGTA</td>
<td>10 Heps</td>
<td>10 Heps</td>
<td>7.4</td>
</tr>
<tr>
<td>0.003 Ca^{2+}</td>
<td>145 NaCl</td>
<td>0.42 CaCl₂/1 MgCl₂</td>
<td>1 Na-HEDTA</td>
<td>10 Heps</td>
<td>10 Heps</td>
<td>7.4</td>
</tr>
<tr>
<td>0.01 Ca^{2+}</td>
<td>145 NaCl</td>
<td>0.72 CaCl₂/1 MgCl₂</td>
<td>1 Na-HEDTA</td>
<td>10 Heps</td>
<td>10 Heps</td>
<td>7.4</td>
</tr>
<tr>
<td>0.03 Ca^{2+}</td>
<td>145 NaCl</td>
<td>0.91 CaCl₂/1 MgCl₂</td>
<td>1 Na-HEDTA</td>
<td>10 Heps</td>
<td>10 Heps</td>
<td>7.4</td>
</tr>
<tr>
<td>0.1 Ca^{2+}</td>
<td>145 NaCl</td>
<td>0.1 CaCl₂/1 MgCl₂</td>
<td>10 Heps</td>
<td>10 Heps</td>
<td>7.4</td>
<td>NaOH</td>
</tr>
<tr>
<td>0.5 Ca^{2+}</td>
<td>145 NaCl</td>
<td>0.3 CaCl₂/1 MgCl₂</td>
<td>10 Heps</td>
<td>10 Heps</td>
<td>7.4</td>
<td>NaOH</td>
</tr>
<tr>
<td>1 Ca^{2+}</td>
<td>145 NaCl</td>
<td>1 CaCl₂/1 MgCl₂</td>
<td>10 Heps</td>
<td>10 Heps</td>
<td>7.4</td>
<td>NaOH</td>
</tr>
<tr>
<td>3 Ca^{2+}</td>
<td>145 NaCl</td>
<td>3 CaCl₂/1 MgCl₂</td>
<td>10 Heps</td>
<td>10 Heps</td>
<td>7.4</td>
<td>NaOH</td>
</tr>
<tr>
<td>10 Ca^{2+}</td>
<td>133 NaCl</td>
<td>10 CaCl₂/1 MgCl₂</td>
<td>10 Heps</td>
<td>10 Heps</td>
<td>7.4</td>
<td>NaOH</td>
</tr>
<tr>
<td>30 Ca^{2+}</td>
<td>103 NaCl</td>
<td>30 CaCl₂/1 MgCl₂</td>
<td>10 Heps</td>
<td>10 Heps</td>
<td>7.4</td>
<td>NaOH</td>
</tr>
</tbody>
</table>
Figure 4.1 Intracellular Ca\textsuperscript{2+} buffering strength affected the activation kinetics of TRPV3 by 2APB.

A and B, TRPV3 transiently transfected HEK293 cells were continuously stimulated with 100 μM 2APB as indicated (gray bars) in whole-cell configuration. Cells were held at 0 mV and voltage ramps from -100 mV to +100 mV were applied to record TRPV3 currents. Intracellular Ca\textsuperscript{2+} was buffered by 10 mM BAPTA (A) or 1 mM EGTA (B), respectively. Extracellular cations were replaced with NMDG\textsuperscript{+} as indicated (black bars) to confirm the cation permeability of TRPV3. I-V curves at indicated time points were shown in the insets.

C and D, Summary (n=5 for EGTA, n=16 for BAPTA) of current densities at 100 s after 2APB application (C) and time constants (\(\tau_{\text{act}}\)) obtained from exponential fits (D) of current developed at -100 and +100 mV using intracellular solutions containing either 1 mM EGTA (open bars) or 10 mM BAPTA (filled bars). Note, logarithmic scale is used in D to accommodate the large difference between data values. *, \(p < 0.05\); †, \(p < 0.001\), different from the data obtained with 1 mM EGTA intracellular solutions.
Figure 4.2 Intracellular Ca\textsuperscript{2+} buffering strength affected sensitization process of TRPV3 induced by repetitive 2APB stimulation.

A and B, Similar to fig 4.1, except 100 µM 2APB was repetitively applied as indicated (gray bars). Intracellular Ca\textsuperscript{2+} was buffered either by 10 mM BAPTA (A) or 1 mM EGTA (B). Insets were I-V curves at indicated time points.

C, Two consecutive 2APB stimulations were spaced with a 10-min interval and the intracellular solution contained 1 mM EGTA.

D, Summary data of peak current densities at -100 and +100 mV for intracellular solutions containing 10 mM BAPTA (circles), 10 mM EGTA (squares), and 1 mM EGTA (triangles), n=6-9.

E, A simulation of Ca\textsuperscript{2+} diffusion from the source in solutions containing 1 and 10mM EGTA or 10mM BAPTA using online PORE program developed by J. Kenton. The initial [Ca\textsuperscript{2+}] at the inner mouth of the pore is arbitrarily assigned to 1 mM.

F, Representative traces of deactivation during the 20-ms step pulse from 0 to -100 mV in the EGTA (green) and BAPTA (black) buffered intracellular solutions.
Figure 4.3 Changes of voltage dependence of TRPV3 during repetitive stimulations.

A and B, TRPV3 expressed HEK293 cells in whole-cell mode were buffered by 1 mM EGTA (A) or 10 mM BAPTA (B). Ramp protocols from -100 mV to +100 mV and step protocols from -100 mV to +200 mV were applied alternatively. Top traces showed currents at -100 mV (open circles) and +100 mV (filled circles) obtained from voltage ramps, and lower traces showed currents obtained from the step protocol with those from -100, 0, +100, and +200 mV highlighted in black.

C, Tail currents from step protocol were normalized and plotted as a function of pre-pulse membrane potentials, giving rise to conductance-voltage relationship (G-V curves). Upper traces were obtained from cells with intracellular Ca\textsuperscript{2+} buffered by 1 mM EGTA and lower traces showed cells with intracellular Ca\textsuperscript{2+} buffered by 10 mM BAPTA.

D, $V_{1/2}$, $z$ (the valence of the gating charge), and voltage-dependent fraction (VDF) of the total activity were compared between two intracellular Ca\textsuperscript{2+} buffering conditions. Blank circles, intracellular solution containing 1 mM EGTA; black circles, intracellular solution containing 10 mM BAPTA. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ different from BAPTA; #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ different from the first stimulation, $n=3-12$. 
Figure 4.4 The intracellular Ca\textsuperscript{2+} effect on TRPV3 was through CaM.
A, Similar to fig 2B, TRPV3 expressed HEK293 cells in whole-cell mode were repetitively stimulated with 100 μM 2APB but some free Ca\textsuperscript{2+} was added into the 10 mM BAPTA containing intracellular solution to make a constant 1.6 μM free Ca\textsuperscript{2+} intracellular solution.
B, Similar to (A) except 20 μM ophiobolin A, a CaM antagonist, was included in the intracellular solution.
C, Summary of peak current densities of TRPV3 at -100 mV (\textit{blank}) and +100 mV (\textit{filled}) in the presence (\textit{circles}, n=11) or absence (\textit{triangles}, n=5) of ophiobolin A.
Figure 4.5 Identification of a CaM binding site in the N-terminus of TRPV3.
A, Fragments of MBP-TRPV3 fusion proteins were tested for binding to CaM in the presence of 50 mM Ca\(^{2+}\). These fragments were designated as a, b, c, etc., and their positions in the full-length TRPV3 are indicated in parentheses. Black, gray, and open bars indicated positive, weakly positive, and negative binding to CaM, respectively. Green bars in the full-length TRPV3 indicated transmembrane segments and the pore loop.

B, Representative binding results showed the sizes and the amounts of \(^{35}\)S-labeled MBP-TRPV3 fusion proteins used in the binding reactions (upper panel) and retained by CaM-agarose in the presence of 50 μM Ca\(^{2+}\) (lower panel).

C, 113RQKKRLKKR\(^{122}\) was mutated to SQAEASDAEG as indicated. The mutated MBP-fusion fragment lost the CaM binding under same assay condition as in (B). This experiment was performed by Jisen Tang.
Figure 4.6 Voltage dependence of TRPV3RK\(^{-}\) mutant in response to repetitive 2APB stimulations.

A, Similar to Fig 4.3A, except that TRPV3RK\(^{-}\) mutant was expressed in HEK293 cells.

B, G-V curves were obtained from cells with intracellular Ca\(^{2+}\) buffered by either 1 mM EGTA (left) or 10 mM BAPTA (right).

C, Similar to Fig 4.3D, No significant difference was found between the EGTA and BAPTA intracellular solutions except for some small changes in VDF. *, \(p < 0.05\) different from BAPTA; #, \(p < 0.05\) different from the first stimulation.

D, Summary data of TRPV3 peak current densities by the first (left) and 23rd (right) 2APB stimulation obtained from cells with intracellular Ca\(^{2+}\) buffered by 1 mM EGTA (open bars) and 10 mM BAPTA (filled bars).
Figure 4.7 Extracellular Ca$^{2+}$ blocked TRPV3.

A, TRPV3-expressed HEK293 cell was stimulated with 100 µM 2APB in the normal bath solution containing 2 mM Ca$^{2+}$. During the stimulation, bath solution was replaced with a Ca$^{2+}$-free bath containing 0.1 mM EGTA and the same concentration of 2APB. Intracellular Ca$^+$ was buffered by 10 mM BAPTA. Left, time course of TRPV3 response; right, I-V curves at indicated time points.

B and C, TRPV3-expressed HEK293 cells were repetitively stimulated with 100 mM 2APB in the absence of extracellular Ca$^{2+}$. Intracellular Ca$^{2+}$ was buffered by either 10 mM BAPTA (B) or 1 mM EGTA (C).

D, Summary of peak current densities of TRPV3 at -100 mV (blank) and +100 mV (filled) in the absence of extracellular Ca$^{2+}$ obtained from cells with intracellular Ca$^{2+}$ buffered by 10 mM BAPTA (circles) and 1 mM EGTA (triangles), n=4-7.

E, Similar to (D) except that intracellular solutions contained 10 mM BAPTA with free Ca$^{2+}$ buffered at 1.6 µM supplemented (squares) or not (diamonds) with 20 µM ophiobolin A, n=5-9.

F, HEK293 cells with (red) or without (black) TRPV3 were loaded with fluo4/AM. 2APB (166 µM) evoked [Ca$^{2+}$]i changes were monitored in the presence (left) or absence (right) of extracellular Ca$^{2+}$. 
Figure 4.8 Extracellular Ca\(^{2+}\) dose-dependently inhibited TRPV3.

A, TRPV3 expressed HEK293 cells were stimulated with 100 \(\mu M\) 2APB for consecutive 6 times. *Upper panel* showed changes in extracellular Ca\(^{2+}\) concentrations in perfusates during six successive applications of 2APB as indicated. *Traces below* showed currents at -100 (open circles) and +100 mV (filled circles) recorded using voltage ramps. Intracellular Ca\(^{2+}\) was buffered with 1 mM BAPTA (see Table 1).

B, I-V curves obtained from the 1\(^{\text{st}}\), 3\(^{\text{rd}}\), and 6\(^{\text{th}}\) stimulation of TRPV3 at different extracellular Ca\(^{2+}\) concentrations.

C, Summary of inhibitory effect of TRPV3 current by external Ca\(^{2+}\) during six consecutive 2APB stimulations. Currents at -100 mV (*upper*) and +100 mV (*lower*) were normalized to that obtained in the Ca\(^{2+}\)-free solution for each stimulation, \(n=4–17\). Two-site formula was used to fit the data as described in the text.

D and E, IC\(_{50}\) values of the high affinity state gradually increased during repetitive stimulation (*D*), while the fraction of high affinity state decreased (*E*).
Figure 4.9 TRPV3D641N mutant abolished the extracellular Ca\(^{2+}\) high affinity inhibition.

A, Similar to fig 4.8 A, except that Asp641 of TRPV3 was mutated to Asn to give rise to TRPV3D641N. *Left*, dose-dependent inhibition of TRPV3D641N by extracellular Ca\(^{2+}\); *right*, I-V curves of TRPV3D641N mutant obtained at different extracellular Ca\(^{2+}\) concentrations.

B, Summary of inhibitory effect of TRPV3D641N current by external Ca\(^{2+}\). Only one affinity state (the lower one) was maintained after the mutation.
Figure 4.10 Effect of intracellular Ca$^{2+}$/CaM binding on the activation of TRPV3D641N mutant.

A and B, Similar to Fig 4.2A and 4.2B, except that TRPV3D641N was expressed and recorded. Intracellular Ca$^{2+}$ was buffered by 10 mM BAPTA (A) or 1 mM EGTA (B).

C, Activation kinetics of TRPV3D641N obtained from cells with intracellular Ca$^{2+}$ buffered by 10 mM BAPTA (black) or 1 mM EGTA (gray). Currents at -100 mV (opened symbols) and +100 mV (filled symbols) were recorded with ramp protocol.

D, Similar to (C), but the RK CaM binding site mutations were introduced into TRPV3D641N background to give rise to TRPV3D641N-RK- double mutant. This double mutant was expressed and recorded.

E and F, Peak current densities (E) and activation time constant (F) were compared between TRPV3D641N and TRPV3D641N-RK-, with intracellular Ca$^{2+}$ buffered by 1 mM EGTA and 10 mM BAPTA as indicated. *, p<0.05; **, p <0.001, n=9-13.

G, Step protocol was used to compare the voltage dependence of the double mutant under two intracellular Ca$^{2+}$ buffering conditions: 10 mM BAPTA (left current traces) and 1 mM EGTA (right current traces). No obvious difference was found for instantaneous currents ($I_1$), steady state currents ($I_{300}$), and tail currents ($I_{tail}$).
The main discovery presented in this chapter has been published in 2008 (Xiao et al., 2008b). Two equal contributors including Rui Xiao and Jinbin Tian performed most experiments for this chapter. Specifically, Rui Xiao made the initial discovery and performed experiments in figure 5.1, 5.2, and 5.7 (Xiao R also prepared all cRNAs for oocyte recordings). Dr. Jinbin Tian performed electrophysiological recordings and provided figure 5.3, 5.4, 5.5, and 5.6. This represents the nature of the collaboratory research in our laboratory.

5.1 Introduction

When we were studying the factors that regulate the functions of TRPV3, an interesting report from Sakata’s group caught our attention. In this study, Asakawa et al. investigated the genetic mutations responsible for the spontaneous autosomal dominant hairless phenotype of DS-Nh mice and WBN/Kob-Ht rats (Asakawa et al., 2006). Strikingly, detailed analysis revealed mutations of a common amino acid Gly573 of the TRPV3 channel for these two
independent hairless rodent strains. In DS-Nh mice, Gly573 of mouse TRPV3 was substituted by Ser, while in WBN/Kob-Ht rats, Gly573 of rat TRPV3 was replaced with Cys (Asakawa et al., 2006). It was also found that WBN/Kob-Ht rats spontaneously developed atopic dermatitis (AD)-like skin rash under conventional husbandry conditions in the presence of *Staphylococcus aureus*. Linkage analysis suggested that the genes responsible for dermatitis and hairlessness were tightly linked and inseparable from each other (Asakawas et al., 2005). Therefore, the TRPV3 mutations at Gly573 appear to be associated with both hair loss and certain forms of dermatitis.

Because there is almost no functional data in Asakawas et al.’s report (Asakawa et al., 2006), we introduced the site-directed mutations of Gly573 to Ser (G573S) and Gly573 to Cys (G573C) into our mouse TRPV3 clone, and studied the mutant functions in detail.

5.2 Expression of G573S and G573C mutants of TRPV3 kills host cells

Initially, we used the same strategy for wild type TRPV3 and constructed the G573S and G573C mutants of TRPV3 into pIRES2-EGFP vectors for bi-cistronic expression, hoping to directly express them in HEK293 cells and record the currents. Surprisingly, one day after transfection, almost no green cells could be found and most cells were dead. We speculated that these two mutants were probably constitutively active and the expression killed host cells by forming unregulated leak channels. In order to bypass this problem, we tagged the N-
termini of the mutants and wild type TRPV3 with EGFP and then constructed the products into a tetracycline-inducible tet-on vector pcDNA4/TO. The host cell type used was T-Rex293 cells.

Before adding doxycycline to induce the expression of the tet-on constructs, the transfection alone did not cause any obvious death in T-Rex293 cells (Fig 5.1 A untreated panels). Twenty-six hours after adding 20 ng/ml doxycycline to induce the expression of the transfected constructs, most cells that expressed GFP-tagged wild type TRPV3 remained healthy morphology and displayed green fluorescence. In contrast, most cells that expressed G573S or G573C mutants were dead (Fig 5.1 A doxycycline-treated panels). Although some GFP expression was still shown for G573S and G573C mutants, almost all green cells were dying and no success was achieved when we tried to establish whole-cell configuration for those green cells.

5.3 Ruthenium red partially rescued host cells expressing G573 mutants

We reasoned that if the host cell death induced by the expression of G573S and G573C mutants of TRPV3 was due to the constitutive opening of the mutant channel, we should be able to rescue host cells by blocking the channel activity. Indeed, we found that inclusion of 10 μM ruthenium red, a nonspecific TRPV channel blocker, in the culture medium promoted the survival of TRPV3 G573S-expressed host cells. Many cells displayed similar GFP signals as wild type TRPV3-expressed host cells in the first 6-8 hours after induction (Fig 5.2
ruthenium red-treated panels). However, longer induction of G573S mutant of TRPV3 still killed most host cells even in the presence of ruthenium red (data not shown). The partial rescuing effect of ruthenium red implies that ruthenium red probably is not able to completely block the mutant channel activity over the entire course of cell culture. Constitutively open cation channels lead to two harmful consequences. First, uncontrolled cation influx breaks the normal membrane potential, and second, large Ca\(^{2+}\) influx elevates the intracellular [Ca\(^{2+}\)]. Constantly high [Ca\(^{2+}\)] is toxic to cells and it causes many downstream signaling events. To test which event was the major reason for host cell death, we cultured cells expressing wild type TRPV3 or G573S mutant of TRPV3 in a medium supplemented with 2 mM EGTA, which should chelate the extracellular Ca\(^{2+}\) concentration to about 0.5 \(\mu\)M, and most Ca\(^{2+}\) influx through TRPV3 should be abolished under this low extracellular Ca\(^{2+}\) culturing condition. The wild type TRPV3-expressing cells displayed a round-up morphology which is typical for HEK293 cells cultured in low extracellular [Ca\(^{2+}\)] solution, but most of them were still alive because GFP signals were readily detected. However, for G573S- and G573C-expressing cells, massive cell death was still present and green fluorescence was hardly seen. Therefore, large Ca\(^{2+}\) influx-induced Ca\(^{2+}\) toxicity is probably not the major reason for the cell death induced by the TRPV3 mutants.

5.4 Constitutive current of G573S and G573C mutants of TRPV3 expressed in Xenopus oocytes
The severe cell death caused by the expression of both G573S and G573C mutants of TRPV3 made it difficult to study the mutant channel properties. Since most mammalian cell cultures including ours are maintained at 37°C which is higher than the temperature threshold of 32 °C for TRPV3 activation, it is possible that the cell culture temperature was responsible for the constitutive activation of the TRPV3 G573 mutants and the consequent cell death. An alternative heterologous expression system is *Xenopus* oocytes, which has an advantage in that oocytes are typically maintained at 19°C, much lower than the temperature threshold of TRPV3. Therefore, we reasoned that we might be able to record the whole-cell current of the TRPV3 mutants using the *Xenopus* oocytes expression system.

Typically, one day after cRNA injection, about 20% wild type TRPV3-expressing oocytes showed some level of discoloring of animal and vegetal poles which was probably due to the damages caused by the injection process. However, about 75% G573 mutants-expressing oocytes displayed obvious discoloring and sick-looking appearance. We performed two-electrode voltage clamp experiments with the remaining healthy ones. Unlike wild type TRPV3 injected or uninjected oocytes, G573 mutants showed large basal current (−30.5 ± 7.6 (n=11) and −32.7 ± 9.1 (n=18) μA at −100 mV, for G573C and G573S, respectively); whereas the basal currents for wild-type TRPV3 injected and control uninjected oocytes were −0.52 ± 0.11 μA (n = 16) and −0.31 ± 0.07 μA (n = 7), respectively (Fig 5.3 A to C for sample traces, and D for summary). Wild-type TRPV3 injected
oocytes were sensitive to the TRPV3 agonist 2APB, and the 2APB induced TRPV3 currents were blocked by 10 μM RR (Fig 5.3 A). Heat, by itself, was not a direct activator of TRPV3 for oocyte expression system because the heat-induced response in TRPV3-expressing oocytes was not different from that in uninjected ones (Fig 5.3 A and C), indicating that other factors present in mammalian cells but not Xenopus oocytes are required for the heat activation of TRPV3 or the activation threshold of TRPV3 is much higher in oocytes than in mammalian cells. Interestingly, despite the large basal currents, G573 mutants of TRPV3 were not sensitive to 2APB but displayed larger heat-evoked response (Fig 5.3 B). Importantly, the basal currents of the mutants were partially blocked by RR, indicating that these currents were indeed TRPV3 currents because leak current was not sensitive to RR. The partial effect of RR to mutants could be due to the change of pore conformation of the G573 mutants, which made the RR block incomplete.

5.5 Ligand-induced response of G573 mutants and wild type TRPV3 expressed in Xenopus oocytes

We also compared the ligand-induced response of wild type TRPV3 and G573 mutants. Camphor, another TRPV3 agonist, was used in this study. Wild type TRPV3 showed strong response to 10 mM camphor, while no camphor-evoked current increase was detected with the G573 mutants or control uninjected oocytes (Fig 5.4 A for sample traces, B for summary). Interestingly, when G573 mutants were co-injected with wild type TRPV3, the response to both camphor
and 2APB was significantly larger than if wild-type TRPV3 was expressed alone in oocytes (Fig 5.4 A for sample traces, and B for summary), indicating that although the G573 mutants were irresponsive to ligand stimulation, their presence, presumably through heteromerization, enhanced the ligand-induced response of the wild type TRPV3.

5.6 Heat-induced response of G573 mutants and wild type TRPV3 expressed in *Xenopus* oocytes

G573 mutants of TRPV3 displayed much larger heat induced response compared with wild type TRPV3 expressed in *Xenopus* oocytes, which led us to speculate that the mutations of G573 decreased the temperature activation threshold of the TRPV3 channel. To quantify the temperature sensitivity of G573 mutants, we first reduced the bath temperature to 10ºC for 2 min to allow the current to stabilize at this low temperature, then the temperature was increased to 40ºC using a temperature ramp (Fig. 5.5 A). Consistent with our previous results, uninjected control and wild type TRPV3-injected oocytes showed little response to heat stimulation up to 40ºC, while G573 mutants of TRPV3 showed large heat-evoked response (Fig 5.5 A and B). Interestingly, the 10ºC cold treatment significantly decreased the basal currents for oocytes that expressed G573 mutants, especially those that coexpressed G573 mutants and wild type TRPV3 together, implying that the basal currents of G573 mutants were indeed due to the room temperature-evoked TRPV3 response.
In the temperature range between 10ºC and 40ºC, the temperature coefficient (Q10) values were 1.245 ± 0.006 for G573S (n=7) and 1.249 ± 0.007 for G573C (n=5), suggesting that the responses of the G573 mutants are due to normal thermodynamic changes rather than temperature sensitivity of the channel within this temperature range. Given that G573 mutants still showed considerable basal current at the temperature as low as 10ºC which was the lowest temperature our perfusion system could achieve, it is possible that the temperature threshold of the G573 mutants of TRPV3 is much lower than 10ºC, making it difficult to determine with the conventional experimental approach.

On the other hand, the current-temperature relationship plot revealed a biphasic response to temperature between 10 and 40ºC for oocytes that coexpressed G573 mutants and wild type TRPV3 (Fig 5.6 A upper right figure), while all others showed near linear relationships. By normalizing the currents to those at 40ºC, the response pattern for each type of injection becomes even clearer (Fig 5.6 B). Because the mutant TRPV3 and wild type TRPV3 co-injected oocytes clearly showed heat-induced response, we plotted the current at −100 mV in the log scale as a function of temperature to determine the Q10 values and the temperature threshold. The two linear portions of the curve were fitted by linear regression (Fig 5.6 C). The temperature threshold was determined to be 15 ± 1ºC, which is significantly lower than published threshold values for wild type TRPV3, ranging from 31 to 39ºC (Peier et al., 2002b; Xu et al., 2002). Thus, the TRPV3 heterotetramer composed of both wild type TRPV3 and G573 mutants displays a
much lower temperature threshold than the wild type, which gives rise to the large basal current and causes cell death in the host cells. However, the temperature regulation of wild type and G573 co-expressed oocytes is rather mild since the $Q_{10}$ value at $>15^\circ C$ was only 3.4, which is significantly smaller than those determined for wild type TRPV1 ($>20$, Vlachová et al., 2003), TRPV2 ($\sim20$, Xu et al., 2002), and TRPV3 ($\sim20$, Xu et al., 2002).

5.7 Expression of wild type TRPV3 partially rescued G573 mutant-expressing host cells

In both DS-Nh mice and WBN/Kob-Ht rats, the hair-less phenotype was due to the autosomal dominant mutation of Gly573 site of TRPV3. Equal amounts of wild type and mutant TRPV3 are expressed in the heterozygote hairless rodents (Asakawa et al., 2006). To mimic this expression pattern, the wild type TRPV3 in pcDNA3 vector was cotransfected with the inducible constructs encoding the GFP-tagged wild type, G573S, or G573C mutant into T-Rex 293 cells. After induction, no obvious increase in cell death rate was observed for GFP-tagged wild type TRPV3 and TRPV3-pcDNA3 co-expressed cells (Fig 5.7 upper panels). Interestingly, the expression of the wild type TRPV3, but not the vector, increased the protein level of the G573 mutants in host cells as judged by the much more intense GFP signals (Fig 5.7 lower panel). However, most cells expressing G573 mutants of TRPV3 were still severely damaged based on changes in cell morphology even with the coexpression of the wild type TRPV3, indicating that under our culture conditions, the expression of the wild type
TRPV3 was not able to fully rescue the host cells expressing G573 mutants although a partial improvement in the expression of the mutant proteins was achieved with the help of the wild type TRPV3. The co-expression experiments with *Xenopus* oocytes had shown that the temperature threshold for the activation of mutant and wild type TRPV3 heterotetramer was about 15ºC; therefore it was possible that wild type and mutants TRPV3 co-expressed in T-Rex293 cells were also strongly activated by cell culture temperature of 37ºC resulting in host cell death. Because keratinocytes from hairless rodents survived with the expression of G573 mutants of TRPV3, and wild type TRPV3 was not shown to be constitutively active and kill native keratinocytes at the body temperature of 37ºC which is already higher than the activation threshold of TRPV3, other endogenous factors might exist in keratinocytes to inhibit TRPV3 function.

5.8 Summary

Mutations in a common site of TRPV3 have been identified for two naturally occurring hairless strains of rodents (Asakawa et al., 2006). In order to understand the underlying mechanism of these mutations responsible for the hairless phenotype, we performed functional studies with these mutations in both mammalian cell and *Xenopus* oocyte expression systems.

When expressed in both HEK293 cells and *Xenopus* oocytes, G573S and G573C mutants of TRPV3 killed host cells presumably by membrane
depolarization due to the constant channel activity. Interestingly, when tested in the Xenopus oocyte expression system, both mutants lose the sensitivities to 2APB and camphor, two chemical ligands of TRPV3. Although the mutants show heat-induced response, a closer examination of the mutants reveals a low $Q_{10}$ value $<2$ between 10-40ºC, indicating that the heat-evoked current increase for the mutants is purely due to thermodynamics. Therefore, G573 mutants of TRPV3 also lose the temperature sensitivity within the temperature range we tested. Two possibilities may explain the loss of temperature and ligand sensitivities: first, currents recorded in oocytes are leak currents which are not expected to be gated by TRPV3 agonists or heat; second, mutant channels in oocytes are already fully active; therefore, no further response is anticipated. Our results support the later one because RR, a TRPV3 blocker, largely blocks the basal current of G573 mutant-injected oocytes and partially rescues the T-Rex293 host cells expressing G573 mutants. If the basal current is a purely leak current, it should not be sensitive to RR. However, since RR only has a partial effect, it is possible that the RR-insensitive currents we recorded are due to the leakage of plasma membrane which is typical for dying cells. Importantly, the co-expression of G573 mutants and wild type TRPV3 recovers the ligand and heat sensitivity of the mutants. Strikingly, mutants and wild type TRPV3 co-expressed cells display much larger ligand- and heat-induced response, again, supporting the view that G573 mutants of TRPV3 are super-active under normal conditions and greatly increase the sensitivity of wild type TRPV3 to ligands and heat, presumably by forming heterotetramers.
TRPV3 is highly expressed in mammalian skin keratinocytes. Hairless phenotype in rodents caused by mutations of a single amino acid Gly573 in TRPV3 indicates the importance of this channel for normal skin function. Dysregulation of TRPV3 channel function seems to be the initial trigger to cause hairless and dermatitis phenotypes in DS-Nh mice and WBN/Kob-Ht rats. Skin allergens such as eugenol have been shown to activate TRPV3 and induce the release of the inflammatory cytokine interleukin-1 (Xu et al., 2006) which can cause dysfunction of skin keratinocytes (Blumberg et al., 2007). Therefore, multiple lines of evidence support that constitutively active G573 mutants of TRPV3 are indeed detrimental to skin keratinocytes. Interestingly, the body temperature of most mammals is higher than the reported temperature activation threshold of TRPV3, which means that TRPV3 should already be active under physiological condition. Since the expression of endogenous TRPV3 has not been associated with cell death, some endogenous factors may exist to inhibit TRPV3 channel function at the body temperature or the expression level of endogenous TRPV3 is relatively low and the constant opening of these channels does not kill host cells, which also explains the fact that keratinocytes from those two hairless rodents strains still survive in spite of damaged functions (Asakawa et al., 2006).

Why a single amino acid substitution causes such a dramatic change of TRPV3 channel function? Based on the sequence alignment, Gly573 is located at the intracellular loop between S4 and S5 of TRPV3. Camphor and 2APB are
structurally unrelated agonists for TRPV3, and dramatic synergistic but not
addictive activation mode exists between these two compounds (Xu et al., 2005),
indicating that they are not acting on the same binding site of TRPV3. Based on
our results that G573S and G573C mutants are not responsive to both 2APB and
camphor, Gly573 is unlikely the binding site for either of them. G573 mutants
also displayed no temperature dependence within 10-40°C; therefore, the
mutations somehow fix the channel in the constant open state and non-
responsive to any further stimuli. S4 is critical for the voltage sensing of all
voltage-gated cation channels; however, most TRP channels lack the positively
charged amino acids found in S4 of voltage-gated cation channels and display no
obvious voltage dependence. Recently, the importance of the S4-S5 linker as an
essential conformational transducer coupling S4 voltage sensor and the pore has
been realized and confirmed (Lu et al., 2002; Long et al., 2005). According to
many functional studies and the X-ray structure determined by MacKinnon and
colleagues, all voltage-gated K⁺ channels are composed of two relatively
independent modules: S1-S4 form the voltage-sensing module, while S5-S6 form
the pore module (Lu et al., 2001; Lu et al., 2002; Jiang et al., 2003; Long et al.,
2005). For Shaker voltage-gated K⁺ channel, the intracellular gating mechanism
involves a direct physical interaction between the S4-S5 linker distal to the
primary voltage-sensor S4 and the C-terminal end of pore-forming S6 segment,
and the short C-terminus of S6 functions as the intracellular gate of Shaker
channel (Lu et al., 2002; Long et al., 2005). Although most TRP channels are not
voltage sensitive, they function as polymodal sensors and respond to multiple
chemical and physical stimuli. Some action sites of TRP channels by their agonists have been determined: for instance, three cysteines in the N-terminus of TRPA1 have been shown to be important for the sensitivity of TRPA1 to multiple chemical reactive reagents (Macpherson et al., 2007; Hinman et al., 2006); while Arg491 in S2, Tyr511 and Ser512 in the transition between the second intracellular loop and S3 region of TRPV1 are the capsaicin binding sites (Jordt & Julius, 2002). Since TRP channel and voltage-gated K⁺ channel both belong to the 6 transmembrane domain cation channel superfamily and some structural similarities have been found between them (Owsianik et al., 2006), it is reasonable to speculate that the S4-S5 linker of TRP channels also function as the conformational transducer coupling the chemical and physical stimuli sensing of TRP channels to the S5-S6 pore. Strikingly, Gly573 in the S4-S5 linker is conserved in all TRP channels and present in the linker region of Shaker, therefore, it is highly possible that Gly573 is critical for the physical interaction between S4-S5 linker and the intracellular gate locating at the C-terminus of S6 of TRP channels.
Figure 5.1 Expression of G573C and G573S mutants of mouse TRPV3 caused severe host cell death.
T-Rex293 cells were transiently transfected with GFP-tagged wt TRPV3 and G573 mutants in pcDNA4/TO vectors. Bright field (middle) and fluorescence (sides) images were acquired to monitor cell morphology change and expression of GFP-tagged constructs. Without induction by doxycyclin, little cell death and green fluorescence could be detected; whereas 1 day after 20 ng/ml doxycyclin treatment, GFP signals were readily detected with wild type TRPV3 transfected cells but not G573 mutants transfected cells. Massive cell death occurred after the induction of G573 mutants but not wild type TRPV3. Fluorescence images were taken using the same exposure time, gain value and other camera settings.
Figure 5.2 The cell-killing effect of G573 mutants of TRPV3 was partially rescued by ruthenium red but not by lowering extracellular \([\text{Ca}^{2+}]\).

A, Similar to fig 5.1, TRex-293 cells were transfected with wild type TRPV3 or TRPV3G573S. Phase contrast (middle) and fluorescence (sides) images were acquired 7 hours after doxycyclin induction. Ruthenium red (RR) at 10 \(\mu\text{M}\) was added in the medium during induction in the right two panels. After RR treatment, more GFP signals and normal-shaped host cells were detected for TRPV3G573S transfected cells.

B, Extracellular \([\text{Ca}^{2+}]\) was lowered by adding 2 mM EGTA. However, severe cell death still occurred and little GFP signal could be detected for TRPV3G573S transfected cells.

Note, GFP-V3G573C had a similar effect as GFP-V3G573S (not shown) for both conditions shown in (A) and (B). Fluorescence images were taken using the same exposure time, gain value and other camera settings.
Figure 5.3 G573 mutants of TRPV3 were constitutively active when expressed in *Xenopus* oocytes.

A, Wild-type TRPV3 injected oocyte was held at -20 mV. Time courses of response were recorded at -100 mV by two-electrode voltage clamp. Shown were the responses of wild type TRPV3 to 300 μM 2APB and heat (40ºC) stimulations (*left*). RR at 10 μM blocked 2APB-evoked TRPV3 current (*right*). I-V curves under indicated conditions were shown in insets.

B, Similar to (A), but TRPV3G573C was injected and recorded. The response of mutant to heat was larger than that of wild type TRPV3, and RR (10 μM) partially blocked the constitutive current of TRPV3G573C.

C, Un-injected oocytes showed no response to 2APB but a similar response to heat (40ºC) stimulation as wild type TRPV3 injected oocytes, indicating that this response was independent of TRPV3 expression.

D, Summary of basal currents at -100 mV for all oocytes. Responses for individual cells were plotted and mean values were indicated by the thick bars. Controls (Cntl) were uninjected oocytes.

In this figure, all cRNAs were prepared by Rui Xiao, and all recordings were performed by Jinbin Tian.
Figure 5.4 Agonists-induced responses of G573 mutants and wild type TRPV3 expressed in *Xenopus* oocytes.

A, Un-injected oocytes or oocytes injected with defined constructs were stimulated with 10 mM camphor. Currents at -100 mV were recorded with two electrode voltage clamp. Insets, I-V curves obtained in the absence (dashed lines) or presence (solid lines) of 10 mM camphor.

B, Summary of currents at -100 mV induced by 10 mM camphor (opened bars) and 300 µM 2APB (filled bars). *p < 0.05 different from control, †p < 0.05 different from WT+G/S, n=4-10. Controls (Cntl) were uninjected oocytes.

In this figure, all cRNAs were prepared by Rui Xiao, and all recordings were performed by Jinbin Tian.
Figure 5.5 Heat-induced responses of G573 mutants and wild type TRPV3 expressed in *Xenopus* oocytes.

A, Un-injected oocytes or oocytes injected with defined constructs were stimulated heat (40°C). Currents at -100 mV were recorded with two electrode voltage clamp. Immediately before a temperature ramp to 40°C, a temperature drop from room temperature (~22°C) to 10°C as indicated was applied to stabilize the basal current. Note, co-injection of wild type TRPV3 and TRPV3G573S significantly increased the response to both 2APB and heat.

B, Representative I-V curves under different chemical or temperature stimulation conditions for oocytes recorded in (A).

In this figure, all cRNAs were prepared by Rui Xiao, and all recordings were performed by Jinbin Tian.
Figure 5.6 Temperature dependence of G573 mutants and wild type TRPV3 expressed in *Xenopus* oocytes.

A, Current-temperature relationships of uninjected oocytes (Cntl) and oocytes injected with defined constructs (TRPV3G573S, wild type TRPV3, and TRPV3G573S + wild type TRPV3).

B, Same set of data as in (A) was normalized to current at 40°C.

C, Log current vs. temperature plot for a representative oocyte coexpressing wild type TRPV3 and TRPV3G573S. The intersection of the two linear fit lines gave the temperature threshold, and Q10 values were determined by the antilog of 10*slope* (10^{10*slope}).

D, Summary of relative response (stimulated current/basal current) induced by temperature ramp from 22°C to 40°C for different groups of oocytes. *, *p < 0.05* different from control; †, *p < 0.05* different from WT+ G/S.

In this figure, all cRNAs were prepared by Rui Xiao, and all recordings were performed by Jinbin Tian.
Figure 5.7 Partial rescue of G573 mutants expression by wild type TRPV3. Similar to Fig 5.1, but wild type TRPV3 in pcDNA3 vector or vector alone were transfected on top of GFP-tagged constructs. Expression of GFP-tagged constructs was induced by 20 ng/ml doxycycline 16 hr post-transfection. Phase contrast (middle) and fluorescence (sides) images were acquired 8 hrs after induction. When GFP-tagged TRPV3G573S was co-expressed with wild type TRPV3, although massive cell death still occurred, more GFP signal could be readily detected compared to cells coexpressing GFP-TRPV3G573S + pcDNA3 vector. Note, GFP-V3G573C had a similar effect as GFP-V3G573S (not shown). Fluorescence images were taken using the same exposure time, gain value and other camera settings.
CHAPTER 6

FUNCTIONAL REGULATION OF TRPC4 and TRPC5

Rui Xiao made the initial discovery and performed most experiments in this chapter with the help of Dr. Michael Zhu.

6.1 Introduction

TRPC4 and TRPC5 channels share high similarity in primary sequences and regulatory mechanisms. Among the TRPC subfamily, TRPC4 and TRPC5 are unique in that both are largely potentiated by 100 μM lanthanides and they display typical double-rectifying “S”-shaped I-V curves with a flat region between 20-40 mV (Plant & Schaefer, 2005), which help distinguish TRPC4 and TRPC5 from other TRP channels. Both TRPC4 and TRPC5 are able to form heterotetramers with TRPC1 \textit{in vitro} and \textit{in vivo}, and the formation of the heterotetrameric channels greatly changes the I-V curves from double-rectifying to strongly outward-rectifying ones.

Most functional studies of TRPC4 and TRPC5 are performed in heterologous expression systems such as HEK293 cells, CHO cells, and \textit{Xenopus} oocytes.
Stimulation of receptors coupled to PLC has been repeatedly reported to activate TRPC4 and TRPC5, with G_{q/11}-coupled histamine receptor H_{1}R, muscarinic receptor M_{3}R and bradykinin receptor B_{2}R activating PLCβ, and receptor tyrosine kinases including EGF receptor, activating PLCγ (Plant & Schaefer, 2005). U73122, a PLC inhibitor, but not its inactive analog U73343, abolishes the response of TRPC4 and TRPC5 to the stimulation by receptor (Schaefer et al., 2000). Therefore, PLC is required for the activation of TRPC4 and TRPC5. Three direct events are downstream of PLC activation: breakdown of PIP_{2}, generation of IP_{3} and DAG. PIP_{2} directly binds to and regulates many TRP channels including TRPV1, TRPM8, TRPM4, and TRPM5. Recently, Zholos' laboratory in collaboration with ours have shown that PIP_{2} specifically inhibits TRPC4α but not TRPC4β by directly binding to the 84 amino acid region in the C-terminus of TRPC4α that is missing in TRPC4β (Otsuguro et al., 2008). Therefore, PLC may activate TRPC4α by the hydrolysis of PIP and releasing the channel from the inhibition by the lipid. However, the importance of PIP_{2} inhibition in TRPC4 and TRPC5 gating is still questionable because TRPC4β, which lacks the inhibition by PIP_{2}, shows a similar response to receptor activation as TRPC4α and the dependence on PLC. Thus, it appears that PIP_{2} plays a modulatory role on the activation of TRPC4α. DAG directly activates TRPC3, TRPC6 and TRPC7 (Hofmann et al., 1999), but it has an inhibitory effect on TRPC4 and TRPC5 by causing the desensitization through activating PKC (Venkatachalam et al., 2003). The binding of IP_{3} to IP_{3}Rs in ER leads to Ca^{2+} release and the depletion of ER
Ca\textsuperscript{2+} store. The activation of TRPC4 and TRPC5 definitely requires intracellular Ca\textsuperscript{2+}. This is supported by the finding that TRPC4 or TRPC5 current could hardly be detected by receptor activation if intracellular [Ca\textsuperscript{2+}] was tightly buffered to below 10 nM (Xiao R, unpublished observation). However, Ca\textsuperscript{2+} by itself is not enough to directly activate TRPC4 and TRPC5. Therefore, it appears that intracellular Ca\textsuperscript{2+} also plays a permissive role for the activation of TRPC4 and TRPC5. The involvement of ER Ca\textsuperscript{2+} store depletion in the activation of TRPC4 and TRPC5 is still under debate, but most heterologous studies do not support the store-operated activation mechanism for TRPC4 and TRPC5.

TRPC4 is widely distributed in various tissues including brain, intestinal smooth muscles, vascular endothelial cells, and etc. whereas TRPC5 is more restricted in the brain. However, very limited conclusive results have been obtained from experiments using native cells expressing TRPC4 or TRPC5. For most reports that claim to have successfully recorded native TRPC4 or TRPC5 currents, the I-V relationship is virtually linear and significantly different from that obtained from heterologous studies, raising the question whether these really represent true TRPC4 or TRPC5 currents. Three major problems exist for recording native TRPC4 and TRPC5 currents. First, both TRPC4 and TRPC5 have been shown to mainly reside intracellularly under resting conditions. Second, in most tissues, TRPC4 and TRPC5 co-express with other TRP channels and possibly form heterotetramers with them, which significantly changes the gating properties of TRPC4 and TRPC5 and makes it difficult to verify the nature of the recorded
current. Third, there is no specific pharmacological tool available to study native TRPC4 or TRPC5 current so far.

As described in above sections, both TRPC4 and TRPC5 are highly expressed in mammalian brains. TRPC4 is highly expressed throughout the frontal cortex, lateral septum (LS), pyramidal cell layer of the hippocampus, dentate gyrus, olfactory bulb, and ventral subiculum (vSUB), while TRPC5 displays high expression levels in the frontal cortex, pyramidal cell layer of the hippocampus, dentate gyrus, and hypothalamus (Fowler et al., 2007). High expression levels of TRPC4 and TRPC5 in the brain suggest the important roles of these channels for normal brain function. Indeed, in hippocampal neurons, TRPC5 has been shown to be highly expressed in the growth cones of hippocampal neurites, and suggested to play a role to maintain the normal neurite length and growth cone morphology (Greka et al., 2003). However, although abundant TRPC4, TRPC5, and TRPC1 are co-expressed in the samata of hippocampal neurons, whole-cell recordings have failed to show TRPC5-like current in hippocampal neurons (Greka et al., 2003).

In vertebrates, the G protein-coupled receptor (GPCR) superfamily contains 1000-2000 members (about 1% of the genome). The superfamily can be divided into two major groups: a) chemosensory GPCRs (>1000 members) including odorent receptors, taste receptors, and pheromone receptors, and b) non-sensory GPCRs (about 400 members) which detect various endogenous signals including
peptides, lipids, neurotransmitters, and nucleotides (Bockaert & Philippe Pin, 1999; Vassilatis et al., 2003). Within non-sensory GPCRs, more than 90% are expressed in brain (Vassilatis et al., 2003). Some GPCRs in brain are coupled to G_{q/11} signaling pathway such as metabotropic glutamate receptor mGluR5 and mGluR1; some GPCRs are G_{i/o}-coupled, including opioid receptors, dopamine receptor D_2R, cannabinoid receptors, somatostatin receptors, and etc.; some others are G_s-coupled, including some serotonin receptors, glucagon family receptors, VIP/PACAP receptors. Among all heterotrimeric G proteins, G_{i/o} proteins are particularly highly expressed in the brain. For example, G_{\alpha_o} accounts for about 0.5% of the total membrane proteins in neurons (Strathmann et al., 1990). However, in contrast to the well characterized signaling pathways transduced by G_{q/11} and G_s, the signal transduction mechanisms of G_{i/o} proteins are poorly known. Studies with G_{\alpha_o} knockout mice have shown that these mice displayed short life span and multiple neurological abnormalities including severe motor impairment and hypersensitivity to pain (Jiang et al., 1998), indicating the important roles of G_{i/o} signaling for normal motor behavior and pain sensation.

Since TRPC4 and TRPC5 are activated by a receptor-operated mode and there are abundant G protein-coupled receptors and receptor tyrosine kinases in the brain, TRPC4 and TRPC5 might be under complex regulation by these receptors.

Although the G_{q/11}-coupled signaling pathway has been shown to activate TRPC4 and TRPC5 in vitro by different laboratories, a possible role of other types of G proteins for regulating TRPC4 and TRPC5 has not been reported before. We are
interested in the potential role of G_{i/o} proteins because they have been shown to be enriched in the growth cones of neurites and regulate neurite outgrowth (Chen et al., 1999), a feature that is also for TRPC5 in hippocampal neurons (Greka et al., 2003).

6.2 Stimulation of μ opioid receptor (μOR) specifically activates TRPC4/C5 but not other TRPCs

Our initial attempt to study the possible regulation of TRPC channels by G_{i/o} signaling pathway faced a problem: after co-transfection of each TRPC channel with G_{q/11}-coupled histamine receptor H_{1}R, surprisingly, only TRPC4 and TRPC5 showed response to histamine stimulation. We found that the transfection reagent lipofectamine 2000 we used in our initial trial had long-lasting inhibitory effect on most TRPC channels except TRPC4 and TRPC5, presumably due to the sensitivities of TRPC channels to liposome. To avoid this problem, we performed transfection using liposome free magnetic nanoparticle-based transfection reagent Polymag (OZ Bioscience). One day after co-transfection with H_{1}R, we found that all TRPC channels except TRPC1 and TRPC2 showed response upon stimulation with 100 μM histamine (Fig 6.1 A to H). The lack of response to histamine for TRPC1 and TRPC2 was somewhat expected because TRPC1 has been reported to mainly localize intracellularly and it requires other TRP channels such as TRPC4 or TRPC5 to be translocated into plasma membrane (Hofmann et al., 2002). The similar reason might also explain the lack
of function for TRPC2. In fact, there are very limited reports on functional studies of TRPC2 in expression systems.

After verifying that the magnetic transfection method work for all TRPC channels, we tested the effect of endogenous G_s-coupled β adrenergic receptor and the transfected G_i/o-coupled μ opioid receptor (μOR) on the activation of each TRPC channel. No response was detected for any TRPC channel when cells were stimulated with β receptor agonist isoproteranol (data not shown), indicating that TRPC channels are not coupled to G_s signaling pathway. However, a weak response of TRPC4 and TRPC5 to 1 μM DAMGO, an agonist of μOR, was clearly seen (Fig 6.2 D and E). The best known function G_i/o signaling pathway is the inhibitory effect to adenylyl cyclases for which they are named after ("i" means inhibitory). In addition, the G_i/o signaling is known to stimulate G protein-gated inwardly rectifying K^+ channels and to inhibit neuronal voltage-gated Ca^{2+} channels, mainly through the G_{βγ} subunits of the heterotrimeric G proteins. In terms of neuronal functions, both effects are considered inhibitory because the net effect of G_i/o activation seems to increase membrane stability and reduce depolarization. Strikingly, in our initial test the activation of G_i/o-coupled receptor μOR led to the opening of TRPC4 and TRPC5. These channels mediate Na^+ and Ca^{2+} influx, causing membrane depolarization and therefore should be an excitatory effect.
In order to confirm that μOR stimulation indeed leads to the activation of TRPC4 and TRPC5, we transfected μOR into stable cell lines expressing each member of the TRPC subfamily. Again, only TRPC4 and TRPC5 displayed strong activation to 1 μM DAMGO treatment (Fig 6.3 A). In fact, the response of TRPC4 and TRPC5 stable cell line to DAMGO was even much stronger than that of TRPC4 or TRPC5 transient transfected cells using Polymag, presumably due to the relatively low transfection efficiency using Polymag. Since lipofectamine transfection method did not affect TRPC4 and TRPC5 functions, we performed all following transfection experiments using lipofectamine. When co-expressed with μOR, both TRPC4 and TRPC5 were dose-dependently activated by DAMGO (Fig 6.3 B and C, and summary data in D), and the EC$_{50}$ value for TRPC4α, TRPC4β, and TRPC5 are $6.33 \pm 1.39$ nM, $1.41 \pm 0.33$ nM, and $1.64 \pm 0.50$ nM, respectively (Fig 6.3 D).

6.3 Other G$_{i/o}$-coupled receptors also operate TRPC4 and TRPC5
In order to test how general the G$_{i/o}$ signaling pathway in the activation of TRPC4 and TRPC5, we examined several other G$_{i/o}$-coupled receptors including serotonin receptor 5HT$_1$AR, muscarinic acetacholine receptor M$_2$R, and dopamine receptor D$_2$R. Serotonin (5HT), carbachol (CCh), and quinpirole, specific agonists for 5HT$_1$AR, M$_2$R, and D$_2$R respectively, all dose-dependently activated TRPC4 and TRPC5 when the channel was co-expressed with their respective receptor (Fig 6.4 A, B, and C). Therefore, in general, the G$_{i/o}$ signaling
pathway is excitatory for TRPC4 and TRPC5 and this effect is independent of receptor types.

Muscarinic acetylcholine receptor M₃R, a G₉/₁₁-coupled receptor, is endogenously expressed in our HEK293 cells. Therefore, CCh-induced TRPC4 and TRPC5 activation in HEK293 cells co-expressing TRP channels and M₂R could be due to the activation of endogenous M₃R instead of the heterologously expressed M₂R. To rule out this possibility, we compared the CCh response of HEK293 cells expressing TRPC5 alone or TRPC5 with M₂R. The expression of Gᵢₒ-coupled M₂R significantly increased the maximal response and left-shifted the dose-response curve to CCh (Fig 6.5 A, TRPC5+M₂R, and 6.5 B for dose-response curve). In contrast, the overexpression of another G₉/₁₁-coupled muscarinic receptor M₅R on top of the endogenous M₃R had a minimal effect on the maximal response, although the dose-response curve to CCh was still greatly left shifted. The change in the apparent affinity to agonist was expected with M₅R overexpression because of the increased receptor density to trigger efficient G₉/₁₁ activation at lower receptor occupancy. However, increased efficacy to CCh stimulation was only seen with the coexpression of M₂R, but not M₅R. Therefore, the excitatory effect of Gᵢₒ-coupled M₂R for TRPC5 activation is indeed different from that induced by G₉/₁₁-coupled M₃R or M₅R. Similar experiments were performed with TRPC4α and TRPC4β (data not shown, but see Otsuguro et al., 2008), and same conclusion was drawn.
6.4 The excitatory effect of G\textsubscript{i/o} signaling pathway to TRPC4 and TRPC5 is PTX sensitive

Pertussis toxin (PTX) specifically blocks the G\textsubscript{i/o} signaling pathway by catalyzing ADP-ribosylation of the \(\alpha\) subunits of the heterotrimeric G proteins including G\textsubscript{i}, G\textsubscript{o}, and G\textsubscript{t}, which prevents the interaction of G proteins with their receptors (Birnbaumer, 1990). We incubated HEK293 cells co-expressing TRPC4/C5 and \(\mu\)OR with 200 ng/ml PTX overnight. TRPC4 and TRPC5 were activated by 100 nM DAMGO and 100 \(\mu\)M CCh sequentially. Strikingly, the responses to DAMGO for both TRPC4 and TRPC5 were almost completely abolished by the PTX treatment, while the responses to CCh was still intact (Figure 6.6 A to C), strongly supporting that the DAMGO-induced TRPC4/C5 activation indeed occur through PTX-sensitive G\textsubscript{i/o}-signaling pathway.

An established feature for TRPC4 and TRPC5 was the translocation to the plasma membrane upon stimulations. We tested the possibility that PTX treatment might deplete TRPC4/C5 proteins in the plasma membrane therefore abolish the response of TRPC4/C5 to DAMGO stimulation. We performed a surface biotinylation assay to measure the amount of TRPC5 proteins expressed on the plasma membrane. As shown in figure 6.6 D, no obvious change in the cell surface expression level of TRPC5 was detected after the PTX treatment; nor did the total TRPC5 level change in the cell lysates. Therefore, the blocking effect of PTX to DAMGO-induced TRPC4/C5 response was not due to the change of protein expression in the cell and on the plasma membrane.
6.5 Confirmation of the excitatory effects of G\textsubscript{i/o}-coupled receptors on TRPC4 and TRPC5 using whole-cell patch clamp recordings

Most of our initial tests of the potential involvement of G\textsubscript{i/o} signaling pathway in the activation of TRPC4/C5 were performed in a semi high-throughput fashion using the FlexStation fluorescence plate reader. In order to directly measure currents and study the biophysical properties of G\textsubscript{i/o}-induced TRPC4/C5 activities in details, we performed whole-cell patch clamp experiments. In order to rule out the involvement of the endogenous G\textsubscript{q/11} signaling pathway, we tested cells co-expressing TRPC4/5 with 5HT\textsubscript{1}AR or \(\mu\)OR. Consistent with our previous results from the FlexStation, the activation of G\textsubscript{i/o}-coupled receptors evoked TRPC4 and TRPC5 currents (Fig 6.7 A to C). Therefore, combining the results from FlexStation measurements and whole-cell recordings, we conclude that G\textsubscript{i/o} signaling pathway is excitatory for the activation of TRPC4 and TRPC5 but not other TRPC channels.

6.6 Synergistic effect between G\textsubscript{q/11} and G\textsubscript{i/o} signaling pathways for the activation of TRPC4 and TRPC5

Interestingly, for most cells co-expressing TRPC4\(\alpha\) and \(\mu\)OR, the activation of endogenous G\textsubscript{q/11}-coupled M\textsubscript{3}R by 100 \(\mu\)M CCh did not induce any current or in rare cases evoked a small outwardly-rectifying current (Fig 6.8 C for sample trace, and D for summary). On the other hand, stimulation by DAMGO alone also did not activate TRPC4\(\alpha\) in most cells tested (Fig 6.8 A for sample traces, and D
for summary) (only 3 out of 8 cells responded to 100 nM DAMGO treatment). Strikingly, for all cells tested, the co-application of DAMGO and CCh always evoked strong activation of TRPC4α (Fig 6.8 A-C for sample traces, and D for summary). We also tested cells co-expressing TRPC5 and μOR, but performed the experiments in a slightly different fashion. Similar to the results shown in Fig 6.8, in some rare cases, DAMGO or CCh stimulation alone activated TRPC5 (Fig 6.9 A and B for sample traces). For many cells tested, neither DAMGO nor CCh alone could evoke TRPC5 current. However, the pretreatment of DAMGO and CCh greatly increased the chance of obtaining the TRPC5 current by sequential CCh and DAMGO stimulation, respectively (Fig 6.9 C for sample traces and D for summary). Therefore, it appears that G\textsubscript{i/o} and G\textsubscript{q/11} signaling pathways are interdependent for a full activation of TRPC4/C5, i.e. there is a synergy between these two G protein signaling pathways in respect to activation of TRPC4/C5.

In FlexStation experiments with HEK293 cells expressing only TRPC4 or TRPC5, TRPC4/C5-mediated depolarization could be readily detected by CCh stimulation of endogenous G\textsubscript{q/11}-coupled M\textsubscript{3}R, although the relative degree of depolarization was not as strong as that induced by G\textsubscript{i/o} signaling pathway (Fig 6.5). Why could we typically not record TRPC4/C5 current evoked by G\textsubscript{q/11} signaling in our whole-cell patch clamp experiments? This discrepancy could be explained by experimental condition differences between whole-cell patch clamp and FlexStation measurements: in whole-cell recording, only isolated single cells were chosen for recording, while in membrane potential measurements with the
FlexStation, a group of attached cells were measured; another major experimental condition difference is that in whole-cell recordings, all diffusible intracellular contents were lost and replaced by the artificial intracellular solution, while they were intact in the membrane potential measurements.

CCh is a common agonist for both Gq/11 and Gli/o-coupled muscarinic acetylcholine receptors. By expressing M2R into HEK293 cells, CCh will activate both the endogenous Gq/11-coupled M3R and the heterologously expressed Gli/o-coupled M2R, thus allowing simultaneous activation of both G protein systems by one agonist. We compared CCh-induced TRPC4α currents for cells co-expressing TRPC4α with or without the Gli/o-coupled M2R. Consistent with our previous experiments, when M2R was not expressed, the activation of endogenous Gq/11-coupled M3R by CCh hardly evoked any TRPC4 current. In rare cases (roughly 20%), 100 μM CCh weakly activated TRPC4 current which showed strong outwardly rectifying I-V curves (Figure 6.10 A for sample traces and D for summary). However, when M2R was co-expressed with TRPC4α, all cells showed strong response to CCh, and I-V curves were significantly different from those obtained without the expression of M2R in that they shifted from strong outwardly-rectifying to double-rectifying and they displayed large inward currents (Figure 6.10 C for sample traces and D for summary). The difference between I-V curves obtained with or without M2R expression indicates that Gli/o signaling pathway is required to fully activated TRPC4/C5. Similar changes in the shape of I-V curves of TRPC4/C5 from partially activated to fully activated
phases were observed by other laboratories (Obukhov & Nowycky, 2008) and our results support that multiple channel activation states exist for TRPC4/C5.

As a control to rule out the possibility that the fully activated TRPC4 with M2R coexpression by CCh was simply a result of increased receptor expression level for CCh but not the activation of the G\textsubscript{i/o} signaling pathway, we coexpressed the G\textsubscript{q/11}-coupled muscarinic receptor M\textsubscript{5}R. Very similar to cells expressing TRPC4\textsubscript{α} alone, only about 20% cells coexpressing TRPC4\textsubscript{α} and M\textsubscript{5}R showed a response to CCh stimulation. For the responding cells, the I-V curves of TRPC4 current were similar to those obtained from cells expressing TRPC4\textsubscript{α} alone (Figure 6.10 B for sample trace, and D for summary). Therefore, the heterologously expressed M\textsubscript{5}R plays a similar role as the endogenous M\textsubscript{3}R for the activation of TRPC4. In this experiment it was not the receptor level but the receptor types that are important for the full activation of TRPC4/C5. Clearly, G\textsubscript{i/o} signaling pathway plays an essential role in TRPC4/C5 channel activation.

6.7 Activation of TRPC4/C5 by G\textsubscript{i/o} signaling pathway does not occur through inhibiting cAMP/PKA pathway

After we have confirmed that G\textsubscript{i/o} signaling pathway was excitatory for TRPC4 and TRPC5 using both whole-cell recording and FlexStation membrane potential assay, we asked which signaling molecules downstream of heterotrimeric G\textsubscript{i/o} proteins were involved in the activation of TRPC4 and TRPC5. G\textsubscript{i/o} proteins mediate downstream events either through G\textsubscript{α/i/o} subunits or G\textsubscript{βγ} subunits. The
best known function of $G_{\alpha/i/o}$ is the inhibitory effect on some adenylyl cyclases and cAMP/PKA signaling pathway, while $G_{\beta/\gamma}$ have various effects on multiple downstream target molecules, such as activating PLC$\beta$, inhibiting adenylyl cyclase I, activating adenylyl cyclase II-IV, activating PI3K, activating PKD, activating G protein-gated inwardly-rectifying K$^+$ channels, and inhibiting N and P/Q type Ca$^{2+}$ channels.

We firstly tested the potential involvement of the best known function of $G_{\alpha/i/o}$ signaling pathway-inhibiting cAMP production. If the reduction of intracellular cAMP level due to the activation of $G_{\alpha/i/o}$ was required to activate TRPC4 and TRPC5, Forskolin, a toxin known to potently activate adenylyl cyclases and increase intracellular cAMP levels independent of G protein-coupled receptors, should be able to override the effect of $G_{\alpha/i/o}$ and block the activation of TRPC4/C5. However, as shown in figure 6.11, forskolin pre-treatment of HEK293 cells co-expressing TRPC4/C5 and 5HT$_1$AR had no effect on 5HT-evoked TRPC4/C5 response. Therefore, the reduction of intracellular cAMP levels due to the active $G_{\alpha/i/o}$ was not involved in the excitatory effect of $G_{\alpha/i/o}$ signaling pathway for the activation of TRPC4/C5.

6.8 PLC is required for the $G_{\alpha/i/o}$-induced TRPC4 and TRPC5 activation
Because the inhibition of cAMP/PKA downstream of $G_{\alpha/i/o}$ is not required for the activation of TRPC4/C5, the $\alpha$ subunits of $G_{\alpha/i/o}$ proteins might not be involved in
our study. As discussed above, G_{βγ} from activated G_{i/o} proteins have multiple targets and PLCβ is one of the identified targets for G_{βγ}. Several TRPC channels including TRPC3, TRPC6, and TRPC7 have been shown to be dependent on PLC for channel activation (Venkatachalam & Montell, 2007). We tested the potential involvement of PLC in the G_{i/o} signaling pathway for the activation of TRPC4/C5. U73122 is a commonly used PLC inhibitor although the mechanism of action is still unknown (Smith et al., 1990). U73343, a structural analog of U73122, has very weak inhibitory effect on PLC and therefore provides a negative control for U73122 (Bleasdale et al., 1990). As shown in Figure 6.12, the PLC inhibitor U73122, but not its inactive analog U73343, strongly blocked TRPC4 current evoked by the activation of the co-expressed μOR. Recently, TRPC4 and TRPC5 have been shown to be sensitive to reducing reagents and direct activation of TRPC5 has been linked to an endogenous reducing peptide-thioredoxin (Xu et al., 2008). U73122 has a highly thiol-reactive reducing moiety which is lacking in U73343 (Otsuguro et al., 2008), therefore it is also possible that the inhibition of TRPC4 current by U73122 was due to a direct interaction to the channel protein instead of inhibiting PLC. To rule out this possibility, we tested another structurally unrelated PLC inhibitor - ET-18-OCH₃, which has no thiol-reactive moiety and selectively inhibits phosphatidylinositol-specific phospholipase C (PI-PLC) but not phosphatidylcholine-specific PLC (PC-PLC) (Cabaner et al., 1999). A similar inhibition of TRPC4 current was observed when ET-18-OCH₃ was applied (figure 6.12 D), indicating that PLC, or more specifically PI-PLC, is indeed involved in the activation of TRPC4/C5 by the G_{i/o} signaling
PLC$\beta$ is best known to be activated by G$\alpha_{q/11}$, and active PLC$\beta$ typically induces Ca$^{2+}$ transient due to the generation of IP$_3$. G$_{\beta\gamma}$ subunits from the G$_{i/o}$ signaling pathway were shown to directly bind to and activate PLC$\beta$2 (Yoshikawa et al., 2001). Since PLC is required for the activation of TRPC4/C5 and G$\alpha_{i/o}$ appeared not to be involved in PLC activation, we reasoned that if G$_{\beta\gamma}$ downstream of $\mu$OR stimulation also activated PLC$\beta$ in our study, we should be able to detect an intracellular [Ca$^{2+}$] elevation. Surprisingly, for HEK293 cells expressing $\mu$OR, DAMGO stimulation did not induce any detectable Ca$^{2+}$ transient, but the subsequent activation of the endogenous G$_{q/11}$-coupled M3R by CCh stimulation evoked a robust [Ca$^{2+}$]$_i$ increase (Fig 6.13 A, upper first trace), suggesting that the activation of G$_{i/o}$-coupled $\mu$OR does not activate the same PLC$\beta$ shared by the G$_{q/11}$-coupled M3R. After the co-expression of TRPC4/C5 channels, DAMGO treatment induced intracellular [Ca$^{2+}$] elevation (Fig 6.13 upper traces), and this Ca$^{2+}$ transient was purely due to Ca$^{2+}$ influx through the opening of TRPC4/C5 because it was abolished by removal of Ca$^{2+}$ from the extracellular solution (data not shown). Interestingly, among all three types of channels tested, TRPC4$\beta$ displayed the largest [Ca$^{2+}$]$_i$ increase (Fig 6.13 upper traces), suggesting that this isoform has the highest Ca$^{2+}$ permeability. Since the DAMGO-evoked Ca$^{2+}$ transient was the result but not the trigger of the opening of TRPC4/C5 channels,
it is most likely that those PLC isozymes that do not cause significant $[\text{Ca}^{2+}]_i$ elevation in HEK293 cells are involved in the activation of TRPC4/C5 evoked by the $G_{i/o}$ signaling pathway.

6.9 Intracellular $\text{Ca}^{2+}$ is required for TRPC4 and TRPC5 activation induced by $G_{i/o}$ signaling

Although the activation of $G_{i/o}$-coupled receptors did not cause any detectable $[\text{Ca}^{2+}]_i$ increase, local $[\text{Ca}^{2+}]_i$ fluctuation could still play an important role for TRPC4/C5 activation, especially given the relatively slower kinetics of membrane depolarization evoked by $G_{i/o}$-coupled than $G_{q/11}$-coupled receptors. Here $\text{Ca}^{2+}$ influx through the opened TRPC4/C5 channels could have a positive feedback effect on further promoting channel activation. To address the question whether $[\text{Ca}^{2+}]_i$ rise is required for TRPC4/C5 activation, we loaded cells with 25 $\mu$M BAPTA-AM before agonist stimulation. BAPTA is expected to buffer the intracellular free $[\text{Ca}^{2+}]$ to a very low level and to prevent $[\text{Ca}^{2+}]_i$ fluctuation. The responses of both TRPC4 and TRPC5 to DAMGO stimulation were largely abolished by BAPTA, with TRPC4$\beta$ displaying the most drastic block (Figure 6.14 A to C), indicating that some levels of intracellular $\text{Ca}^{2+}$ and or $[\text{Ca}^{2+}]_i$ fluctuation is still required for the activation of TRPC4/C5 by $G_{i/o}$ signaling pathway.

Surprisingly, the activation of TRPC4/C5 by CCh-stimulated $G_{q/11}$-coupled $M_3$R was not affected by BAPTA-AM pre-treatment, suggesting that in contrast to $G_{i/o}$ pathway, $G_{q/11}$ signaling pathway activates TRPC4/C5 independent of a rise in intracellular $\text{Ca}^{2+}$. Taken together, $G_{i/o}$ and $G_{q/11}$ signaling pathways recruit
different PLC isoforms and display distinct intracellular Ca^{2+} dependence for the activation of TRPC4 and TRPC5.

6.10. PKG/cGMP, PKC, and PI3K are not involved in the coupling of G_{i/o} pathway to TRPC4/C5 activation

In addition to PLC, we also tested two other signaling pathways reported to be important for the regulation of TRPC channels. Phosphoinositide 3-kinases (PI3K) and Rac-1 have been shown to be critical for the rapid translocation of TRPC5 (Bezzerides et al., 2004). Because G_{i/o} subunits have been repeatedly reported to directly activate PI3K (Viard et al., 1999; Maier et al., 2000), we reasoned that PI3K might be important in the activation of TRPC4/C5 by G_{i/o} signaling pathway. Wortmannin is a fungal metabolite that selectively and potently blocks PI3K by covalent modification of Lys802 which is involved in the phosphate transfer reaction (Wymann et al., 1996). As shown in figure 6.16, TRPC4β and 5HT_{1AR} coexpressing cells were pretreated with 5 nM wortmannin before any agonist stimulation. Surprisingly, wortmannin-treated cells showed similar response level as untreated control cells to both 5-HT and CCh (Fig 6.15 A for sample trace and B for summary). We also tested another PI3K inhibitor, LY294002. At 10 μM, LY294002-treated cells also displayed similar response as untreated control cells for both 5-HT and CCh, indicating that PI3K is not important for the activation of TRPC4/C5.
We also tested the potential involvement of PKC and PKG for the activation of TRPC4/C5 by the G_{i/o} signaling pathway, because several TRPC channels have been reported to be negatively regulated by these two kinases (Venkatachalam et al., 2003; Kwan et al., 2006; Kuan et al., 2004). Two commonly used PKC specific inhibitors, Ro 31-8220 (IC_{50} = 10 nM) and Go 6976 (IC_{50} = 7.9 nM), were tested. Consistent with previous results that PKC was involved in the desensitization of TRPC5 and played an inhibitory effect on TRPC5 (Venkatachalam et al., 2003; Zhu et al., 2005), for TRPC4β and 5-HT₁AR co-expressing cells pretreated with 1 μM Ro 31-8220 or Go 6976, the response to 5HT was significantly increased (Figure 6.15 C and D). PKG/cGMP pathway has been shown to inhibit TRPC3/C6 (Kwan et al., 2006; Kuan et al., 2004). We tested the effect of LY83583 (an inhibitor of soluble guanylate cyclase with an IC_{50} value of 2 μM) and KT5823 (a selective inhibitor of PKG with an IC_{50} value of 234 nM), no obvious effect on TRPC4 and TRPC5 activation was observed for either drug (data not shown), suggesting that PKG/cGMP signaling pathway was not critical for the excitatory effect of G_{i/o} on the activation of TRPC4/C5.

6.11 Summary
Among the TRPC subfamily, TRPC4 and TRPC5 are particularly highly expressed in the mammalian brain. Functionwise, they are uniquely activated/potentiated by lanthanides at micromolar range. Previous prevailing view about TRPC channel is that all TRPC channels are tightly coupled to PLC through G_{q/11} or receptor tyrosine kinases. We confirmed that the activation of
TRPC4 and TRPC5 requires PI-PLC and $G_{q/11}$ signaling is excitatory for TRPC4 and TRPC5. However, an interesting observation about the roles of $G_{q/11}$ signaling for TRPC4/C5 activation is that the chance to record TRPC4/C5 whole-cell current is rather low if only $G_{q/11}$ signaling pathway is activated. On the other hand, the non-metabolizable GTP analog, GTPγs, is most widely used in various studies to activate TRPC4 or TRPC5. GTPγs non-specifically activates all G protein signalings, therefore, we cannot rule out the involvement of other G protein signalings under this condition.

In our study, we found that $G_{i/o}$ signaling, typically considered as “inhibitory”, played a novel excitatory effect for the activation of TRPC4 and TRPC5. This excitatory effect of $G_{i/o}$ signaling is independent of receptor type. Given the high expression levels of $G_{i/o}$-coupled receptors in the brain, $G_{i/o}$ pathway may play an excitatory effect using TRPC4/C5 as the target. More importantly, $G_{i/o}$ and $G_{q/11}$ pathways play synergistic effect on TRPC4/C5, suggesting that TRPC4/C5 may function as coincident detectors of these two important G protein signaling pathways. As described before, multiple activation statuses exist for TRPC4 and TRPC5. TRPC4/C5 currents evoked by $G_{q/11}$ pathway typically display strongly outward rectification with very little inward current. However, when both $G_{q/11}$ and $G_{i/o}$ signaling are activated, TRPC4/C5 display double rectifying currents with very large inward currents. The consequence of these two distinct activation statuses could be rather significant because under physiological conditions, the inward currents are the most important. Little inward currents of TRPC4/C5
induced by $G_{q/11}$ pathway indicate that very limited $Ca^{2+}$ influx and depolarization can be triggered by $G_{q/11}$ signaling. However, the large inward current typically observed with the activation of $G_{i/o}$ pathway will cause large $Ca^{2+}$ influx and strong membrane depolarization. Both events have significant downstream effects for neurons. Plenty of studies have revealed the depolarization of neurons independent of voltage-gated ion channels (Henley & Poo, 2004). TRPC4 and TRPC5 may underly some forms of these depolarization events in the brain because 1), TRPC4 and TRPC5 are essentially non-voltage-gated cation channels highly expressed in the neurons of mammalian brain; 2), TRPC4 and TRPC5 are coupled to both $G_{q/11}$ and $G_{i/o}$ signaling, and the receptors coupled to these two G proteins are abundantly expressed in the brain; 3) the opening of TRPC4/C5 could induce large $Ca^{2+}$ influx and membrane depolarization.

We studied the potential involvement of signaling events downstream of active heterotrimeric $G_{i/o}$ proteins. It appears that the classical inhibitory role of $G\alpha_{i/o}$ is not important for the $G_{i/o}$-evoked TRPC4/C5 activation because the forskolin treatment failed to affect the TRPC4/C5 response to active $G_{i/o}$. In contrast, PI-PLC downstream of $G_{j}^{\beta\gamma}$ is clearly required for $G_{i/o}$-induced TRPC4/C5 response. Therefore, it is possible that the free $G_{j}^{\beta\gamma}$ subunits from $G_{i/o}$ are important for the excitatory effect of $G_{i/o}$ in terms of TRPC4/C5 activation. However, at this stage we are still not sure about the precise role of $G_{j}^{\beta\gamma}$ subunits for TRPC4 and TRPC5 because 1), PI-PLC downstream of $G_{j}^{\beta\gamma}$ subunits should cause $Ca^{2+}$ release from
ER Ca\(^{2+}\) store, but the activation of G\(_{\text{i/o}}\)-coupled receptors failed to cause any detectable [Ca\(^{2+}\)]\(_i\) increase; and 2), G\(_{\beta\gamma}\) subunits could also come from active G\(_{q/11}\) pathway, but G\(_{q/11}\) and G\(_{i/o}\) pathways play distinct role on the activation of TRPC4/C5 in terms of channel activation probability, kinetics, and strength. Moreover, we cannot rule out the involvement of G\(_{\alpha i/o}\) simply based on lack of effect of forskolin treatment because it is possible that G\(_{\alpha i/o}\) may have other unknown downstream effectors in addition to adenylyl cyclases. More studies are required to elucidate the exact role of G\(_{\alpha i/o}\) and G\(_{\beta\gamma}\) for TRPC4/C5 activation.

Our findings suggest that TRPC4 and TRPC5 are coupled to both G\(_{q/11}\) and G\(_{i/o}\) pathways. Therefore, similar to other TRP channels, TRPC4 and TRPC5 also function as polymodal sensors. In contrast to the fact that most thermo-TRPs integrate multiple environmental stimuli and endogenous inflammatory factors, TRPC4 and TRPC5 are coincident detectors of two important endogenous G proteins signaling under normal conditions. Therefore, TRPC4 and TRPC5 may play an important role for the signal transduction in mammalian neurons under regular conditions.

Taken together, we have revealed a novel excitatory effect of G\(_{i/o}\) signaling for the activation of TRPC4 and TRPC5, two most abundant TRPC channels in the mammalian brains. TRPC4 and TRPC5 may integrate both G\(_{i/o}\) and G\(_{q/11}\) pathways and provide voltage-independent depolarization forces for neurons that express TRPC4/C5.
Figure 6.1 TRPC channels were functionally expressed using magnetic nanobeads-based transfection method. A, G_{q/11}-coupled histamine receptor H1R and pcDNA3 vector were co-expressed in HEK293 cells using nanoparticle-based transfection reagent PolyMag (OZ Bioscience). Histamine (100 μM) was added at indicated time point. Relative fluorescence unit (RFU) was measured to represent the membrane potentials. B to G, H1R was co-expressed with each member of the TRPC subfamily (TRPC1 to TRPC7) using the same transfection method as in (A). Except TRPC1 (B) and TRPC2 (C), all other TRPC channels showed response to 100 μM histamine.
Figure 6.2 TRPC4 and TRPC5 but not other TRPC channels were coupled to G_{i/o} signaling pathway.
A to G, Similar to fig 6.1, but G_{i/o}-coupled opioid receptor μOR was co-expressed with each TRPC member. Except TRPC4 (D) and TRPC5 (E), all other TRPC channels did not induce any depolarization in response to 1 μM DAMGO, a μOR agonist.
Figure 6.3 DAMGO dose-dependently activated TRPC4 and TRPC5.

A, HEK293 cells stably expressing TRPC channels were transfected with μOR. DAMGO (1 μM) was added as indicated (green bars). Membrane potentials of cells were measured using a voltage-sensitive fluorescent dye. TRPC4 and TRPC5 showed strong depolarization upon DAMGO stimulation, while all other TRPC channels were not sensitive to DAMGO.

B and C, As indicated (green bars), different concentrations of DAMGO were applied to cells expressing TRPC4α + μOR (B) and TRPC5 + μOR (C).

D, Summary of dose-dependent activation of TRPC4α (red triangles), TRPC4β (green diamonds), and TRPC5 (black circles) by DAMGO when TRPC4 or TRPC5 was coexpressed with μOR. Relative responses were normalized to that obtained with 1 μM DAMGO and all data were fitted with Hill equation.
Figure 6.4 TRPC4 and TRPC5 were activated by G<sub>i/o</sub> signaling pathway.

A, TRPC4<sub>α</sub> and G<sub>i/o</sub>-coupled serotonin receptor 5HT<sub>1</sub>AR were co-expressed in HEK293 cells. Cells were loaded with FLIPR-membrane potential dye, and membrane potentials of TRPC4<sub>α</sub> and 5HT<sub>1</sub>AR co-expressed cells were monitored as described in material and method section. Serotonin (5HT) was added as indicated (green bar) starting from 100 μM. Upper, sample traces displaying the 5HT-induced membrane depolarization. Lower, summary of dose-dependent activation of TRPC4<sub>α</sub> by 5HT. Responses were normalized to that obtained with 100 μM 5HT, and all data points were fitted with Hill equation, n=5.

B and C, Similar to (A), except that TRPC4<sub>β</sub> and G<sub>i/o</sub>-coupled dopamine receptor D<sub>2</sub>R (B) or TRPC5 and G<sub>i/o</sub>-coupled muscarinic acetylcholine receptor M<sub>2</sub>R (C) were co-expressed. D<sub>2</sub>R specific agonist quinpirole and M<sub>2</sub>R agonist CCh dose-dependently activated TRPC4<sub>β</sub> (B) and TRPC5 (C), respectively. n=3 (B) and 6 (C).
Figure 6.5 Effects of Gq/11 and Gi/o signaling pathways for the activation of TRPC5.

A, TRPC5 alone was expressed in HEK293 cells. CCh was added as indicated (green bar) to activate endogenous Gq/11-coupled muscarinic receptor M3R. Shown are sample traces of TRPC5 opening-induced membrane depolarization upon stimulation by indicated CCh concentrations.

B to D, Similar to (A), but another Gq/11-coupled muscarinic receptor M5R (B), Gi/o-coupled M2R (C), and M2R+M5R (D) were co-expressed with TRPC5. CCh at different concentrations were added as indicated (green bars). Note, the over-expression of muscarinic receptors increased the potency of CCh for the activation of TRPC5.

E, Summary of does-dependent activation of TRPC5 by CCh with or without the co-expression of muscarinic receptors: no muscarinic overexpression (circles), M5R (triangles), M2R (diamonds), and M2R+M5R (squares). All data points were fitted with Hill equation, n=3-6. Note, the over-expression of Gq/11-coupled M5R caused a left-shift of dose response curve of CCh but not the maximal response, whereas Gi/o-coupled M2R caused a left-shift of dose response curve as well as an increase in maximal response level.

F, Comparison of EC50 values of CCh under the four conditions: TRPC5 alone (gray bar), TRPC5+M5R (blue bar), TRPC5+M2R (red bar), and TRPC5+M2R+M5R (purple bar). *, p < 0.01 different from cells that expressed TRPC5 alone.
Figure 6.6 Activation of TRPC4/5 by G<sub>μ/ο</sub>-coupled μOR was PTX sensitive.

A, TRPC5 and μOR co-expressing HEK293 cells were incubated with or without 200 ng/ml PTX overnight. DAMGO (1 μM) and CCh (1 mM) were added as indicated to activate TRPC5. PTX treatment almost completely abolished DAMGO-evoked TRPC5 activation while it partially inhibited the CCh-evoked TRPC5 response. Left, sample traces of TRPC5 response to DAMGO and CCh in the presence or absence of PTX. Right, summary of DAMGO-induced TRPC5 activation with or without PTX treatment. n = 6, *, p<0.05 different from the response of TRPC5 without PTX treatment.

B and C, Similar to (A), except that TRPC4α (B) or TRPC4β (C) was co-expressed with μOR. DAMGO (100 nM) and CCh (250 μM) were added as indicated. n = 8 (B) or 5 (C), *, p<0.05 different from the responses in the absence of PTX treatment.

D, TRPC5 expressed on plasma membrane was labeled with Sulfo-NHS-LC biotin as described in the material and method section. No obvious change was detected between PTX-treated or untreated samples on western blot for TRPC5 in the total cell lysate and biotinylated fractions which represent the TRPC5 protein on plasma membrane. SA-Ag prec: streptavidin-agarose precipitated fraction.
Figure 6.7 Gi/o signaling pathway activated TRPC4 and TRPC5 currents in whole-cell experiments.

A, TRPC5 and Gi/o-coupled 5HT1AR were co-expressed in HEK293 cells. Cells were voltage-clamped at 0 mV in whole-cell configuration. Voltage ramps from -100 mV to +100 mV were applied. Upper, representative time course of TRPC5 response at -100 mV (open circles) and +100 mV (filled circles). Lower, I-V curves obtained at indicated time points. 5HT (10 μM) was added as indicated (green bar).

B and C, Similar to (A), except that TRPC4α + μOR (B) or TRPC4β + μOR (C) were co-expressed. DAMGO at 100 nM was added as indicated (green bars).
Figure 6.8 TRPC4α currents activated by Gq/11 and Gi/o signaling pathways.
A to C, TRPC4α and μOR were co-expressed in HEK293 cells. Cells were held at 0 mV and voltage ramps from -100 mV to +100 mV were applied. DAMGO at 100 nM (red bars) or CCh at 100 μM (blue bars) were added as indicated. Upper, representative time courses of TRPC4α response to DAMGO, CCh, or DAMGO + CCh. Lower, I-V curves of TRPC4α in response to DAMGO (red), CCh (blue), or DAMGO + CCh (black). Base lines are shown in green. Note, many cells did not respond to either DAMGO or CCh alone, but the combination of DAMGO and CCh always evoked a large TRPC4α current which was represented by the very large inward currents.

D, Summary of TRPC4α current densities in response to CCh, DAMGO, or CCh + DAMGO at both -100 mV (left) and +100 mV (right). Note, a synergistic effect exists between DAMGO and CCh for the activation of TRPC4α. In order to display the diversity of the TRPC4α responses at -100 mV (left) and +100 mV (right), individual data points were overlaid with means ± SEM (horizontal lines and open boxes).
Figure 6.9 TRPC5 currents activated by Gq/11 and Gi/o signaling pathways.
A to C, Similar to figure 6.8, but TRPC5 and μOR were co-expressed in HEK293 cells. DAMGO at 100 nM (green bars) or CCh at 100 µM (blank bars) were added as indicated. Left, representative time courses of TRPC5 response to DAMGO (A), CCh (B), or DAMGO + CCh (C). Right, I-V curves of TRPC5 at indicated time points. Base lines are shown in green. Note, only ~20% cells responded to either DAMGO or CCh alone, but the pretreatment of DAMGO or CCh greatly potentiated TRPC5 response to the subsequent CCh or DAMGO stimulations (C).
D, Summary of TRPC5 response at -100 mV to CCh and DAMGO without (blank bar) or with pretreatment of DAMGO (gray bar) and CCh (black bar), n=7. *, p<0.05 different from the responses without pre-stimulation (blank bars)
Figure 6.10 CCh-evoked TRPC4 currents in cells without or with G\textsubscript{\alpha/o}-coupled M\textsubscript{2}R.

A, TRPC4\textsubscript{α} was expressed in HEK293 cells and endogenous M\textsubscript{3}R was activated by 100 µM CCh as indicated (green bar). Few cells (<1/4) showed response to CCh stimulation and shown is an example of responding ones. Upper, time courses of membrane currents at -100 mV (open circles) and +100 mV (filled circles). Lower, I-V curves before stimulation (red) and at the peak of CCh response (black).

B and C, Similar to (A), except that G\textsubscript{q/11}-coupled M\textsubscript{5}R (B) or G\textsubscript{\alpha/o}-coupled M\textsubscript{2}R (C) was coexpressed with TRPC4\textsubscript{α}. Note, for cells co-expressing TRPC4\textsubscript{α} + M\textsubscript{5}R, few of them (<1/4) showed response to CCh and shown is an example of responding one (B). In contrast, all cells co-expressing TRPC4\textsubscript{α} + M\textsubscript{2}R displayed strong response to CCh (C).

D, Summary of TRPC4\textsubscript{α} peak currents in response to 100 µM CCh for cells expressing TRPC4\textsubscript{α} alone (n = 8), TRPC4\textsubscript{α} + M\textsubscript{5}R (n = 12), TRPC4\textsubscript{α} + M\textsubscript{2}R (n = 13). In order to display the diversity of the TRPC4\textsubscript{α} responses at –100 mV (left) and +100 mV (right), individual data points were overlaid with means ± SEM (horizontal lines and open boxes).
Figure 6.11 Effect of forskolin on the activation of TRPC4 and TRPC5.  
A, TRPC4β and 5-HT₁AR were co-expressed in HEK293 cells. 5-HT (10 μM) and CCh (250 μM) were added as indicated. For cells pretreated with 10 μM forskolin (pink), the response of TRPC4β to the activation of both Gᵯₒ-coupled 5-HT₁AR and Gₒ₁₁-coupled M₃R was not different from cells without forskolin treatment (black).  
B, Summary of TRPC4β and TRPC5 activation in response to 10 μM 5-HT without (blank bars) or with (black bars) the pretreatment of 10 μM forskolin, n=6.
Figure 6.12 PLC was required for the excitatory effect of Gi/o signaling pathway for TRPC4/C5.

A, TRPC4β and μOR were co-expressed in HEK293 cells. DAMGO (100 nM) was continuously applied for about 8 min as indicated (green bar). Upper, time course of TRPC4β response are shown at -100 mV (open circles) and +100 mV (filled circles). Lower, I-V curves obtained before DAMGO application (a), at the peak of the response (b), and at the end of DAMGO stimulation (c). Note, desensitization of TRPC4β occurred during this long period of stimulation.

B, Similar to (A), but PLC inhibitor U73122 (5 μM) was added as indicated (orange bar) together with DAMGO for the last 4 min of drug application. Extracellular cations were replaced by NMDG+ (blue bar) to confirm that the constantly active current was due to TRPC4β but not leakage. Note, U73122 almost completely blocked TRPC4β current at both -100 mV and +100 mV.

C, Similar to (B), but U73343 (5 μM), an ineffective structural analog of U73122, was added as indicated (yellow bar) together with DAMGO for the last 4 min. No obvious inhibition of TRPC4β current was observed due to U73343 treatment.

D, TRPC4α and μOR were co-expressed in HEK293 cells. A PI-PLC specific inhibitor (structurally unrelated to U73122), ET-18-OCH3 (10 μM) was added as indicated (red bar) together with DAMGO for the last 3 min of drug application. Note, similar results were confirmed with both TRPC4 and TRPC5.
Figure 6.13 Activation of G\textsubscript{il0} signaling pathway did not induce detectable Ca\textsuperscript{2+} release from ER stores.

A, \(\mu\)OR was co-expressed without or with TRPC4/C5. Cells were loaded with Fluo4/AM and FLIPR membrane potential (FMP) dye simultaneously. Shown are sample traces of Ca\textsuperscript{2+} transients evoked by 100 nM DAMGO and 100 \(\mu\)M CCh in the absence (blue lines) or presence (red lines) of 200 ng/ml PTX overnight treatment. When no TRP channel was expressed, the activation of \(\mu\)OR by DAMGO caused no detectable [Ca\textsuperscript{2+}]	extsubscript{i} elevation, while CCh induced a large [Ca\textsuperscript{2+}]	extsubscript{i} transient. For cells co-expressing TRPC4/C5 and \(\mu\)OR, DAMGO treatment evoked [Ca\textsuperscript{2+}]	extsubscript{i} transient which were due to Ca\textsuperscript{2+} influx through the opening of TRPC4/C5 channels. The DAMGO-induced [Ca\textsuperscript{2+}]	extsubscript{i} increase was completely abolished by PTX treatment.

B, Same set of cells was measured except that membrane potential but not intracellular [Ca\textsuperscript{2+}] was read by the FlexStation. DAMGO strongly activated TRPC4 and TRPC5. Again, DAMGO induced membrane depolarization was completely blocked by PTX treatment.
Figure 6.14 Activation of TRPC4/C5 by G\textsubscript{\textsubscript{\textit{i/o}}} signaling pathway required intracellular Ca\textsuperscript{2+}.

A, HEK293 cells co-expressing TRPC4\textsubscript{\textalpha} and \mu OR were loaded with FLIPR membrane potential dye. In order to chelate intracellular free Ca\textsuperscript{2+} and reduce [Ca\textsuperscript{2+}], cells were incubated with 25 \mu M BAPTA-AM before drug stimulations (pink lines). DAMGO (100 nM) and CCh (250 \mu M) were added as indicated. Upper, sample traces of TRPC4\textsubscript{\textalpha} response without (blue line) or with (pink line) BAPTA-AM loading. Lower, summary of peak response of TRPC4\textsubscript{\textalpha} to DAMGO without (blue bar) or with (pink bar) BAPTA-AM loading. N=9, *, p<0.05 different from control.

B and C, Similar to (A), but TRPC4\textsubscript{\textbeta} (B) and TRPC5 (C) were co-expressed with \mu OR. The response of TRPC4\textsubscript{\textbeta} to DAMGO was completely abolished by BAPTA-AM loading, while CCh-evoked response remained. For TRPC5, DAMGO evoked TRPC5 response was largely blocked by BAPTA-AM loading. N=9 for both (B) and (C), *, p<0.05 different from control.
Figure 6.15 Excitatory effect of G\textsubscript{i/o} signaling pathway for TRPC4/C5 did not involve PI3K, PKC.

A, TRPC4\(\beta\) and 5HT\(_1\)AR were co-expressed in HEK293 cells. 5HT (10 \(\mu\)M) and CCh (100 \(\mu\)M) were added as indicated and membrane potentials were measured. Shown are sample traces of TRPC4\(\beta\) in response to 5HT and CCh in the absence (control, blue line) or presence of 5 nM wortmannin (pink line) or 10 \(\mu\)M LY294002 (red line) pretreatment.

B, Summary of TRPC4\(\beta\) response to 5HT without (blue bar) or with wortmannin (pink bar) or LY294002 (red bar), n=3.

C, Similar to (A), except that cells were pretreated with PKC inhibitors Ro 31-8220 (1 \(\mu\)M) (red line) or Go 6976 (1 \(\mu\)M) (pink line) pretreatment.

D, Summary of TRPC4\(\beta\) response to 5HT without (blue bar) or with Ro 31-8220 (pink bar) or Go 6976 (red bar) pretreatment, n=3. *, \(p<0.05\) different from control.

Note, the PKC inhibitors only enhanced the response to 5-HT, which is consistent with the finding that PKC increases desensitization of TRPC4/C5.
CHAPTER 7

DISCUSSION AND FUTURE PERSPECTIVE

The results from our experiments with TRPV3 strongly support that TRPV3 channel function can be up-regulated and down-regulated by various intracellular and extracellular factors. Thus, similar to other TRP channels, TRPV3 is also a polymodal sensor. Our experimental data with TRPV3 expressed in both mammalian cells and *Xenopus* oocytes confirmed that AA, together with other poly-unsaturated fatty acids, greatly potentiated TRPV3 channel function independent of its downstream signaling in *vitro*. Other groups have shown that many mammalian TRP channels are regulated by various lipids (Hardie, 2003; Nilius et al., 2007). Interestingly, a set of 20-carbon PUFAs including eicosapentaenoic acid (EPA) and AA have been shown to activate TRPV channels in *C. elegans* sensory neurons in *vivo*. Moreover, modulation of TRPV channels by these PUFAs is required for the AWA olfactory signaling and ASH avoidance response of nose touch of *C. elegans* (Kahn-Kirby et al., 2004). Given that TRPV3 is also highly expressed in mammalian tongue and nasal epithelium (Xu et al., 2006), it is possible that TRPV3 also plays a role in taste and olfactory sensations of mammals. PUFAs are commonly used in our daily diets. We
expect that some of them will up-regulate TRPV3 function and modulate our
terpretation of food flavor during food consumption. TRPV3 is also highly
expressed in skin keratinocytes (Chung et al., 2004). During acute tissue injury,
AA and other fatty acids are released in response to inflammation. Up-regulated
TRPV3 function might be involved in the hyperalgesia during tissue injury.

Given that PUFAs activate TRP channels in both C. elegans and Drosophila
(Chyb et al., 1999; Kahn-Kirby et al., 2004), it appears that the regulation of TRP
channels by PUFAs is conserved throughout evolution. In contrast to the
mechanism of action of AA on TRPV1 and TRPV4, potentiation of TRPV3 by AA
does not require the downstream metabolites of AA. PUFAs activate ion
channels either by direct interaction or through indirect effect due to changes of
membrane properties (Patel et al., 2001). The insertion of PUFAs into membrane
sometimes mimic detergent effect, but this non-specific effect can be ruled out
because palmitic and elaidic acids are in-effective for TRPV3 function. Besides,
since linoelaidic acid, a trans-isomer of unsaturated fatty acids which is not
supposed to increase membrane fluidity also potentiated TRPV3, membrane
fluidity change due to PUFA insertion seems not to be critical for the potentiation
effect of PUFAs for TRPV3. Among all PUFAs that potentiate TRPV3 function,
those with the double-bond starting at the fifth position from the carboxylated
head group showed the strongest effect, supporting that a common binding site
might exist in TRPV3 channel protein to directly interact with PUFAs.
Several lines of experiments can help us better understand the significance of PUFA regulation of TRPV3. In TRPV3 deficient mice, only temperature sensitivity of knockout mice has been studied (Moqrich et al., 2005). We can take advantage of TRPV3 knockout mice to study the potential involvement of TRPV3 in sensing PUFAs in daily diet and tissue injury. Besides, more detailed structural-function studies are required to elucidate the molecular mechanism of PUFA action on TRPV3.

TRPV3 channel also displays an intrinsic sensitization property which is independent of stimulation type (Xu et al., 2002; Peier et al., 2002b; Moqrich et al., 2005). Our results showed that calcium inhibits TRPV3 from both intracellular and extracellular sides. During repetitive or prolonged 2APB stimulation, somehow the Ca$^{2+}$ block of TRPV3 is gradually removed, thus giving rise to the gradually increased TRPV3 response. We identified a novel CaM-binding site in the C-terminus of TRPV3. Disruption of this CaM-binding site almost completely abolished the initial intracellular Ca$^{2+}$ inhibition and caused a readily sensitized TRPV3 channel, supporting that CaM regulation is critical for the sensitization property of TRPV3. If intracellular Ca$^{2+}$ inhibits TRPV3 through CaM, a question arises: during repetitive or prolonged stimulations, the opening of TRPV3 should introduce more Ca$^{2+}$ influx into host cells, which supposedly should cause more inhibition to the channel but we only observed gradually increased channel function. This discrepancy could be explained by the selective Ca$^{2+}$ sensing of TRPV3. For any defined single ion channel, intracellular Ca$^{2+}$ signals can be
further divided into two categories (shown in figure 7.1A): Ca$^{2+}$ influx through the opening of ion channel itself causes a transient but intense local [Ca$^{2+}$] spikes up to 100 $\mu$M (local Ca$^{2+}$ signal) (Tadross et al., 2008); Ca$^{2+}$ release from intracellular Ca$^{2+}$ stores or Ca$^{2+}$ influx through other distant ion channels generates global [Ca$^{2+}$] increase up to 5 $\mu$M (global Ca$^{2+}$ signal) (Neher, 1998). Importantly, the local Ca$^{2+}$ signal is restricted within nanodomain of the channel itself due to the buffering by various Ca$^{2+}$-binding proteins. Our proposed model is shown in figure 7.1B: in naïve state, the inhibitory motif within the N-terminus of TRPV3 is close to the channel pore and the pre-associated CaM inhibits TRPV3 channel function. During stimulations, TRPV3 undergoes a gradual conformational change and the inhibitory CaM binding site moves away from the nanodomain of the local Ca$^{2+}$ signal. When 10 mM BAPTA was used as the intracellular Ca$^{2+}$ chelator, local Ca$^{2+}$ signaling was restricted within 10 nM from the channel pore. In contrast, when 1 mM EGTA was used as the intracellular Ca$^{2+}$ chelator, local Ca$^{2+}$ could travel much further; therefore the inhibition of TRPV3 with intracellular solution that contained 1 mM EGTA is more pronounced. For a fully sensitized TRPV3 channel, the inhibitory N-terminal motif moves away from the local Ca$^{2+}$ signaling environment, thus the large Ca$^{2+}$ entry through channel opening has no effect on the CaM-binding site in the N-terminus of TRPV3.

We studied the intrinsic sensitization property of TRPV3 by taking advantage of a synthetic compound 2APB. Arguments might arise because 2APB is not an
endogenous agonist for TRPV3. Since TRPV3 in response to heat also displays sensitization feature (Xu et al., 2002; Moqrich et al., 2005), in the future we should also confirm the similar loss of sensitization to repetitive heat stimulation with our TRPV3 mutants. A potential technical challenge to study heat-induced sensitization of TRPV3 is that plasma membrane is usually somewhat damaged during heat treatment which compromises the seal quality of patch clamp recording and limits the number of heat stimulations. Therefore, we typically could not challenge cells with heat for many times before the seal breaks. An alternative way to confirm our findings is to study an endogenous activator for TRPV3, but so far no endogenous agonist for TRPV3 has been identified. On the other hand, although we found that Ca\textsuperscript{2+} plays an essential role in the sensitization process of TRPV3, we could not rule out the involvement of other factors. Our CaM binding site mutant still showed some extent of sensitization despite much larger initial response compared with wild type TRPV3. It is possible that other CaM binding sites or unknown inhibitory factors are still present to play an inhibitory effect on TRPV3. It is also possible that during repetitive or prolonged stimulations, some facilitatory factors are released to promote the gradually increased TRPV3 response. For example, we have found in our early study that PUFAs potentiate TRPV3 function by increasing channel open probability.

Our studies with TRPV3 revealed that TRPV3 is positively regulated by PUFAs and negatively regulated by Ca\textsuperscript{2+}. Thus, similar to TRPV1 and other well-
characterized TRP channels, TRPV3 is also a polymodal sensor. When originally cloned, TRPV3 was found to be highly expressed in skin. As the first defense system of mammalian bodies, skin provides the primary protection and detects various environmental factors. The polymodal sensor feature of TRPV3 may play an important role for normal skin function. Recently, the underlying genetic basis for two independent strains of hairless rodents has been mapped to the mutations of a common single amino acid Gly573 of TRPV3. We have characterized the functional consequence of the mutants. It turned out that both G573C and G573S mutants were constitutively active and caused severe host cell death. Surprisingly, both mutants lost the sensitivity to heat, 2APB, and camphor, which are well-documented stimuli of wild type TRPV3, suggesting a fully activated TRPV3 by these two mutations. Ruthenium red, a non-specific TRP channel blocker, partially relieved the cell-killing effect of the mutants. Moreover, when wild type TRPV3 was co-expressed with the mutant, the resulting heterotetrameric channels re-gained the heat and 2APB sensitivity but with a much lower temperature threshold than that of wild type TRPV3, indicating the super-active channel functions due to the mutations.

Gly573 is located in the intracellular loop between S4 and S5 (Asakawa et al., 2006). Why do the mutations of this single amino acid transform a closed channel to a fully opened one? The most direct and useful information will come from the detailed structural study of these mutants, but so far virtually no progress has been made due to the technical challenge. Because S4-S5 loop of
voltage-gated potassium channels has been shown to be important for coupling the voltage-sensing motif to the channel pore (Lu et al., 2002; Long et al., 2005), given that TRP channels and voltage-gated K+ channels all belong to the six transmembrane channel superfamily (Clapham et al., 2003), it is possible that Gly573 of TRPV3 may also play a role in coupling the stimuli-sensing motif of TRPV3 to the channel pore. Gly573 and its neighboring amino acids may function like the handle of a door. The conformational changes caused by various physical and chemical stimuli form the force to open the channel “door” through the handle. Mutations of this important amino acid somehow change the conformation of the handle and cause a constitutively open channel insensitive to stimuli. Obviously, more structural-functional studies are required in order to examine this hypothesis. Specifically, studies with point mutations of neighboring amino acids could provide more information about the “handle” function of this region. Besides, G573C and G573S are naturally occurring mutations with obvious phenotypes. We could also mutate this Gly into other amino acids and then examine the consequences. In voltage-gated K+ channels, S4-S5 loop physically interacts with the C-terminal end of S6 and this interaction is thought to be the coupling between voltage sensing and pore opening (Lu et al., 2002; Long et al., 2005). If Gly573 in the S4-S5 loop of TRPV3 is involved in a similar coupling, it is expected that this site is important for the interaction between S4-S5 loop and the C-terminal end of S6. If this is the case, we should be able to identify the region at the C-terminal end of S6 as the binding partner of S4-S5 loop of TRPV3.
In a separate study, we revealed that G_{i/o} pathway, typically regarded as an inhibitory pathway, plays a novel excitatory effect on the activation of TRPC4 and TRPC5. We confirmed our results with four distinct G_{i/o}-coupled receptors: \( \mu \)OR, 5HT\(_1\)AR, M\(_2\)R, and D\(_2\)R. When co-expressed with TRPC4 or TRPC5, but not other TRPC channels, the activation of above receptors all induces strong activation of TRPC4 and TRPC5 in both fluorescence membrane potential assay using FlexStation and whole-cell electrophysiological recordings. Importantly, G\(_{i/o}\) signaling pathway activates TRPC4 and TRPC5 in a synergistic manner with the well-documented G\(_{q/11}\) signaling pathway that has been known for TRPC channel activation. In contrast to the previously prevailing view that all TRPC channels are tightly coupled to G\(_{q/11}\) signaling pathway, we find that G\(_{q/11}\) pathway is much less effective than G\(_{i/o}\) pathway in terms of activating TRPC4 and TRPC5. Since abundant G\(_{i/o}\)-coupled receptors, G\(_{i/o}\) proteins, and TRPC4 and TRPC5 are expressed in mammalian brain (Karasinska et al., 2003; Fowler et al., 2007), it is possible that G\(_{i/o}\) signaling pathway plays an excitatory role under certain conditions. Our results support that full activation of TRPC4 and TRPC5 requires a synergistic action between G\(_{i/o}\) and G\(_{q/11}\) pathways. Therefore, TRPC4 and TRPC5 may function as coincident detectors of two distinct signaling pathways in brain. In mammalian hippocampus, many neurotransmitters are able to activate distinct receptor subtypes coupled to either G\(_{i/o}\) or G\(_{q/11}\) pathway (for example, glutamate activates both G\(_{i/o}\)-coupled mGluR2 and G\(_{q/11}\)-coupled mGluR5). Because both TRPC4 and TRPC5 are highly expressed in hippocampal neurons,
it is expected that a single agonist glutamate should be able to fully activate TRPC4 and TRPC5. However, no TRPC4- or TRPC5-like whole-cell current has been reported from hippocampal neurons. The reason for this failure could be due to first, TRPC4 and TRPC5 proteins are mostly intracellular, would not be accessible by whole-cell recording; second, TRPC4 and TRPC5 are mostly expressed in growth cones of neurites. Whole-cell recordings of growth cones have proven to be rather challenging (Greka et al., 2003); third, there are some unknown inhibitory factors in vivo to prevent the activation of TRPC4 and TRPC5.

In addition to the confirmation of the excitatory effect of G\textsubscript{i/o} signaling pathway for TRPC4 and TRPC5, we also performed a series of functional studies trying to identify the downstream underlying mechanism for G\textsubscript{i/o}-mediated activation of TRPC4 and TRPC5. Similar to other TRPC channels (Venkatachalam & Montell, 2007), PI-PLC is still required for TRPC4 and TRPC5 activation induced by G\textsubscript{i/o} signaling pathway. Since PLC\textsubscript{β} is one of the known downstream targets of G\textsubscript{βγ}, it is possible that G\textsubscript{βγ} mediates TRPC4 and TRPC5 activation following the stimulation of G\textsubscript{i/o}. However, activation of G\textsubscript{i/o}-coupled receptors does not cause any detectable intracellular Ca\textsuperscript{2+} release, which argues against the involvement of PLC\textsubscript{β} because PLC\textsubscript{β} is well-known to cause Ca\textsuperscript{2+} release from ER. Therefore, more experiments are required to clarify the detailed mechanism.

The following questions and experiments are very important in order to fully understand the G\textsubscript{i/o}-induced TRPC4 and C5 activation:
First, which subunit of heterotrimeric \( G_{\alpha i/o} \) proteins are involved, \( G_{\alpha i/o} \) or \( G_{\beta \gamma} \)? As described in previous chapters, all heterotrimeric G proteins are composed of two functional modules-\( G_{\alpha} \) subunit and \( G_{\beta \gamma} \) dimers. We have tested some well-known downstream targets for \( G_{\alpha i/o} \) and \( G_{\beta \gamma} \) (for instance, PKA/cAMP, PLC, PI3K and etc.). It appears that the classical inhibitory function of \( G_{\alpha i/o} \) on adenylyl cyclases is not involved in the excitatory action of the \( G_{i/o} \) pathway. In contrast, PLC downstream of \( G_{\beta \gamma} \) has been shown to be required, indicating that \( G_{\beta \gamma} \) is the primary effector for \( G_{i/o} \) pathway in terms of activating TRPC4/C5. However, the lack of \( Ca^{2+} \) release upon activation of \( G_{i/o} \)-coupled receptors argues against the involvement of a typical PLC. Thus, it is still possible that \( G_{\alpha i/o} \) activates some atypical PI-PLC through an unknown mechanism. Several approaches can help us understand the exact role of \( G_{\alpha i/o} \) or \( G_{\beta \gamma} \): (a) if \( G_{\alpha i/o} \) or \( G_{\beta \gamma} \) directly activate TRPC4 and TRPC5 channels, we can perfuse membranes with purified \( G_{\alpha i/o} \) or \( G_{\beta \gamma} \) proteins in inside-out configuration and record single channel activities of TRPC4/C5. Limitation for this experiment is that if downstream signaling cascade is required, \( G_{\alpha i/o} \) or \( G_{\beta \gamma} \) proteins may fail to activate TRPC4/C5 because all cellular contents are lost in inside-out patches. (b) One can add \( G_{\alpha i/o} \) or \( G_{\beta \gamma} \) proteins in whole-cell electrode and dialyze the cell with \( G_{\alpha i/o} \) or \( G_{\beta \gamma} \) in the whole-cell configuration. Effect of \( G_{\alpha i/o} \) or \( G_{\beta \gamma} \) will be judged by the activation of TRPC4/C5 currents. (c) One can overexpress \( G_{\beta \gamma} \) or constitutively active \( G_{\alpha i/o} \) by transfection. If either one of them is involved in channel activation, changes of basal TRPC4/C5 currents will be expected. (d) By
knocking-down the expression of a specific endogenous $G_{\alpha i/o}$ or $G_{\beta\gamma}$ subunit, if it is critical for the activation of TRPC4/C5, the channel activity will be greatly reduced or eliminated.

Second, why is PI-PLC is required but no Ca$^{2+}$ release was detected? As shown in previous chapters, PI-PLC is required for $G_{i/o}$ pathway-induced TRPC4/C5 activation. The lack of detectable Ca$^{2+}$ release could be due to the limit of sensitivity of our instrument or some atypical PI-PLCs involved in the action of $G_{i/o}$ pathway for TRPC4/C5 do not cause Ca$^{2+}$ release. To address this question, we could use a more sensitive Ca$^{2+}$ imaging instrument to measure any putative local Ca$^{2+}$ release upon activation of $G_{i/o}$-coupled receptors. If still no Ca$^{2+}$ release could be detected, we should consider the possible role of each PI-PLC isozymes (there are totally 11 PI-PLC isozymes including four PLC$\beta$, two PLC$\gamma$, four PLC$\delta$, and one PLC$\varepsilon$).

Third, what is the molecule(s) downstream of PI-PLC that is required for TRPC4/C5 activation? At this point, we only know that PI-PLC and basal [Ca$^{2+}$]$_i$ are necessary, we should also consider downstream signaling molecules involved in this signaling cascade.

Fourth, what is the detailed relationship between $G_{i/o}$ and $G_{q/11}$ signaling pathways in terms of activating TRPC4 and TRPC5? Is there any common mechanism underlying these two pathways or they are independent for the activation of TRPC4 and TRPC5? Synergistic effect between $G_{i/o}$ and $G_{q/11}$ pathways exists for activating TRPC4/C5. Interestingly, PLC$\beta$ is downstream of both $G_{q/11}$ and $G_{i/o}$ pathways, but $G_{q/11}$ and $G_{i/o}$ pathways apparently have distinct
roles for the activation of TRPC4 and TRPC5 with $G_{i/o}$ pathway having much stronger excitatory effect than $G_{q/11}$ pathway. Therefore, some other factors in addition to PI-PLC from $G_{i/o}$ pathway must contribute to the strong excitatory effect.

Fifth, do our discoveries also apply to in vivo systems? All our in vitro studies with TRPC4 and TRPC5 in this thesis were performed with HEK293 expression system. We should confirm our findings with native tissues or cells expressing TRPC4 or TRPC5 endogenously.

Sixth, what is the motif within TRPC4 and TRPC5 molecules that is responsible for the $G_{i/o}$ pathway sensitivity? As discussed above, $G_{q/11}$ signaling pathway appears to be excitatory for all TRPC channels. However, the novel excitatory effect of $G_{i/o}$ signaling pathway is specific for TRPC4 and TRPC5 but not any other TRPC channels. Therefore, there must be some unique structural motif within TRPC4 and TRPC5 that are responsible for this phenomenon. We can compare the sequence of TRPC4/C5 with other TRPC channels. If there is any apparent unique segment, construction of chimera by swapping different segments between TRPC4/C5 and other TRPCs (such as TRPC6) may provide some useful insight on intermolecular domain(s) involved in sensing $G_{i/o}$ signaling.
Figure 7.1 Proposed model for TRPV3 during sensitization.

A. For any defined single Ca\textsuperscript{2+} permeable ion channel, Ca\textsuperscript{2+} signaling is composed of two categories. Local Ca\textsuperscript{2+} signaling comes from the Ca\textsuperscript{2+} influx through the channel protein itself. This \([\text{Ca}^{2+}]_{i}\) transient could reach up to 100 \(\mu\text{M}\) but is restricted within a nanodomain with very fast decay kinetics. In contrast, global Ca\textsuperscript{2+} signaling comes from Ca\textsuperscript{2+} influx through distant ion channels and Ca\textsuperscript{2+} release from intracellular Ca\textsuperscript{2+} stores. This \([\text{Ca}^{2+}]_{i}\) transient could go up to 5 \(\mu\text{M}\) and has slow decay kinetics.

B. The inhibitory CaM-binding site in the N-terminal of TRPV3 is close to the channel pore when channel is in naïve state. During repetitive or prolonged stimulations, this inhibitory motif of TRPV3 gradually moves away from the channel pore and is no longer under the regulation by local Ca\textsuperscript{2+} signaling.
BIBLIOGRAPHY


Physiol Rev. 85(1): 373-422.


Rohacs T, Lopes CM, Michailidis I, Logothetis DE. (2005) PI(4,5)P(2) regulates the activation and desensitization of TRPM8 channels through the TRP domain. Nat Neurosci. 8: 626-634.


shares high homology with transient receptor potential calcium channel proteins. Cancer Res. 61(9): 3760-9.


