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NHERF MEDIATED ASSOCIATIONS WITH ACTIN CYTOSKELETON ARE INVOLVED IN ACTIVATION AND REGULATION OF TRPC4 CHANNEL

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in Molecular Cellular and Developmental Biology Program of the Ohio State University

By

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Receptor-stimulated activation of phospholipase C is accompanied with the release of Ca\(^{2+}\) from internal stores and the influx of Ca\(^{2+}\) from extracellular space. Ca\(^{2+}\) influx is mediated by store-operated channels (SOCs), which are thought to become activated when the internal Ca\(^{2+}\) stores are emptied. However, the exact mechanism that regulates the activity of the SOCs is not well understood. A secretion-like coupling mechanism involving actin-mediated insertion of new channels onto plasma membrane has been proposed for SOC activation. Transient receptor potential canonical proteins (TRPC) are postulated to form SOC. My research focuses on examining the secretion-like coupling hypothesis using murine TRPC4 as an example. In \textit{in vitro} binding studies I showed that TRPC4 as well as phospholipase C (PLC) interacts with the first PDZ domain of Na\(^+\)/H\(^+\) exchanger regulatory factor (NHERF) through C-terminal T/(S)XL motif. NHERF is known to interact with actin-binding ezrin-radixin-moesin (ERM) proteins. Using HEK-293 cell lines stably expressing mouse TRPC4, I showed by coimmunoprecipitation that wild type but not the C-terminal TRL motif deleted mutant TRPC4 is associated with ERM proteins and with actin. Stimulation of PLC by a muscarinic receptor agonist, carbachol, enhanced the association of TRPC4 with ERMs.
and with actin by 100%. Using immunofluorescence labeling and western blotting analysis of externally epitope-tagged TRPC4, I showed that there is about 2-fold increase in the amount of TRPC4 on the cell surface after the carbachol stimulation. Therefore, TRPC4 can form signaling complex with PLC via interactions with NHERF, which in turn links the lipase and the channel to the actin cytoskeleton. Furthermore, TRPC4 is trafficked and inserted into the plasma membrane through regulated association with actin and ERMs upon activation of PLC. My data provide the first physical evidence for the secretion-like coupling hypothesis.
Dedicated to my family
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ABBREVIATIONS

ABP, AMPA receptor binding protein
ATP, adenosine triphosphate
AMPA, α-amino-3-hydro-5-methyl-4-isoxazole propionate
BDNF, brain derived neurotrophic factor
BoNT, botulinum neurotoxins
Bp, base pair
BSA, bovine serum albumin
CAP70, CFTR-binding protein of 70 kDa
CaM, calmodulin
CCE, capacitative Ca^{2+} entry
CCh, carbachol
CFTR, cystic fibrosis transmembrane conductance regulator
CHO, Chinese hamster ovary
CIF, calcium influx factor
CIRB, CaM and IP_{3}R binding
Co-IP, co-immunoprecipitation
COS, cv1 of simian cells
DAB, 3, 3’-diaminobenzidine
DAG, diacylglycerol
DMEM, Dulbecco’s modified Eagle’s medium
DTT, 1,4-dithiothreitol
EBP50, ERM-binding phosphoprotein of 50 kDa
ECL, Enhanced Chemiluminescence
ER, endoplasmic reticulum
ERG, electroretinograph
ERM, ezrin/radixin/moesin
GRIP, glutamate receptor interacting protein
GST, glutathione S transferase
HA, hemagglutinin
HEK, human embryonic kidney
HPSS, Hepes-buffered physiological saline solution
HRP, horse radish peroxidase
HSG, human submandibular gland

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I_{CRAC}, Ca^{2+} release activated Ca^{2+} current
INAD, inactivation no after potential D
IP_{3}R, inositol 1,4,5-trisphosphate receptor
IRES, internal ribosomal entry site
JAS, jasplakinolide
LGCC, ligand gated cation channel
mTRPC4, murine TRPC4
NHE3, type 3 Na^{+}/H^{+} exchanger
NHERF, Na^{+}/H^{+} exchanger, regulatory factor
NINAC, neither inactivation nor afterpotential C
NMDA, N-methyl-D-aspartate
NORPA, no-receptor-potential A
NP-40, nonidet P-40
NTP, nucleotide triphosphate
NSF, N-ethylmaleimide-sensitive fusion protein
OAG, 1-oleoyl-2-acetyl-sn-glycerol
PBS, phosphate-buffered saline
PDGFR, platelet-derived growth factor receptor
PDZ, PSD95/DLG/ZO-1
Ph, phalloidin
PKA, protein kinase A
PKC, protein kinase C
PICK1, protein interacting with C-kinase 1
PLC, phospholipase C
PMSF, phenylmethylsulphonyl fluoride
PNK, polynucleotide kinase
RIPA, radio-labeling immunoprecipitation assay
RyR, ryanodine receptor
PKC, protein kinase C
PLC, phospholipase C
RACE-PCR, rapid amplification of complimentary ends by polymerase chain reaction
RBL, rat basophilic leukemia
RT, reverse transcription
SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOC, store-operated channel
TBST, Tris-buffered saline Tween-20
TG, thapsigargin
TRPC, transient receptor potential canonical family
VGCC, voltage-gated calcium channel
VNO, vomeronasal organ
YAP65, Yes-associated protein of 65 kDa
ZP, zona pellucida
CHAPTER 1

INTRODUCTION

1.1. The significance of intracellular Ca$^{2+}$ signaling and mechanisms to regulate intracellular Ca$^{2+}$ concentration

Ca$^{2+}$ is a versatile and important intracellular messenger emerged from the course of evolution. Calcium signaling operates during the entire life span of the cell: it begins at fertilization triggering new life, continues with development and differentiation and ends with cell death [Berridge et al., 1999]. The influence of Ca$^{2+}$ spreads over the most important activities of cells: ranging from regulating enzyme activities to coupling between excitation and contraction or secretion. The essential requirement of Ca$^{2+}$ as a messenger is that Ca$^{2+}$ must be maintained at very low concentration (~10$^{-7}$ M) at resting state inside the cell. However, cells are surrounded by an environment with a total concentration of free Ca$^{2+}$ at the mM level, which is about 10,000-fold higher than that inside the cell. Therefore, intracellular Ca$^{2+}$ concentration must be strictly regulated. Cells keep the low intracellular Ca$^{2+}$ concentration by two main methods: calcium binding proteins in the cytoplasm and Ca$^{2+}$-ATPases (Ca$^{2+}$ pumps) on the membrane of the calcium reservoirs: the calcium stores inside the cell (endoplasmic or sarcoplasmic
reticulum, ER/SR) and the extracellular milieu [Sorrentino et al., 2001]. To increase intracellular free Ca$^{2+}$ levels, cells mobilize Ca$^{2+}$ from internal stores by calcium-releasing channels, either ryanodine receptor (RyR) or inositol 1,4,5-trisphosphate receptors (IP$_3$R) [Mikoshiba, 1997], or allow Ca$^{2+}$ influx from extracellular environment through channels on the plasma membrane.

There are many channels on the plasma membrane that permit the penetration of Ca$^{2+}$ into the cytoplasm down its concentration gradient. The channels can be divided into three major groups depending on the mechanisms controlling the transition between the open and closed conformations: (1) Voltage-gated Ca$^{2+}$ channels (VGCC) are gated by membrane depolarization. These channels are highly calcium selective. (2) Ligand-gated cation channels (LGCC) are gated through ligand binding. In this type of channels, ligand-binding activates the receptor-formed channel [ionotropic receptors, such as α-amino-3-hydro-5-methyl-4-isoxazole propionate (AMPA) receptors]. (3) Store-operated channels (SOC) that are activated by the depletion of ER/SR calcium stores through a mechanism known as capacitative calcium entry (CCE) [Putney et al., 2001, Parekh et al., 1997]. Related to CCE, there are channels activated by signaling events that ultimately lead to store depletion. However, these channels may or may not be activated by store depletion per se. Therefore, they are collectively called receptor-operated channels (ROCs). ROCs are different from LGCC in that the channels are not receptors themselves. Rather, they are activated by steps downstream for receptor activation.
For different cell types, there are different calcium channels. In excitable cells, such as neuronal, muscle and endocrine cells, there are a wide variety of VGCC, LGCC and SOCs. In nonexcitable cells like blood cells and endothelial cells, the most commonly observed calcium entry is mediated through the SOCs. It is proposed that, in this process, hormones or growth factors bind to the receptors on the cell membrane and activate the phospholipase C (PLC)-mediated pathway, in which PLC hydrolyzes phophatidylinositol 4,5-bisphosphate (PIP2) to produce diacylglycerol (DAG) and IP₃. A biphasic increase in intracellular Ca²⁺ concentration occurs: the first phase results from the binding of IP₃ to IP₃R leading to transient release of Ca²⁺ from internal calcium stores; the second phase results from calcium influx through calcium channels on the plasma membrane activated by the store depletion. The hypothesis was supported by the demonstration of a well-characterized SOC current, the Ca²⁺ release activated Ca²⁺ current (I_{CRAC}) in mast cells and T lymphocytes [Lepple-Wienhues et al., 1996]. I_{CRAC} is a highly Ca²⁺ selective current, has a unitary conductance well below 1 pS and is activated by calcium release from intracellular stores. Different store-depletion-activated currents have been reported in vascular endothelium and epidermal cancer cells displaying a diversity of cation selectivity and conductance characteristics [Vaca et al., 1994; Luckhoff et al., 1994]. The CRAC channel and other SOC channels may represent a family of related Ca²⁺ entry channels that are involved in calcium homeostasis and signaling in diverse cell types. However, the molecular basis of SOC is not clear. The identities of channel components of SOCs and other ROCs has been studied widely. Recently, vertebrate homologues of
the *Drosophila melanogaster* transient receptor potential (TRP) protein have emerged as the most likely candidates for the molecular composition of SOCs.

1.2. TRP channels are proposed to form store-operated calcium channels

1.2.1 Discovery of *Drosophila* TRPs

TRP was originally discovered in *Drosophila* visual transduction system. In contrast to vertebrate photoreceptors (rod and cone cells) whose activation leads to decreased level of cGMP by phosphodiesterase, closure of cyclic-nucleotide-gated (CNG) channels and hyperpolarized receptor potential, the phototransduction in *Drosophila* rhabdomeric photoreceptors is coupled to PLC via a Gq-like protein, and induces a depolarized receptor potential through opening of calcium- or cation-selective channels in the plasma membrane [Hardie, 2001]. The identification of TRP as a Ca$^{2+}$ influx channel activated downstream of PLC stimulation stemmed from genetic analysis in *Drosophila*. Many mutants affecting *Drosophila* vision have been identified in genetic screens using either electroretinograph (ERG) or behavioral assays, such as phototaxis [Montell, 1997]. The *transient receptor potential* (*trp*) mutant displayed a number of biophysical deficits in light responses: showing a significant reduction in response amplitude, lacking a main route of calcium entry, presenting single photon response consistent with the loss of a large number of ion channels, being unable to sustain a steady-state of the receptor potential in response to light, which led to premature termination of the light response and transient inactivation (thus the name *transient receptor potential*) [Hardie and Mink,
Cloning of *trp* gene and later rescue experiments showed that *trp* encodes a Ca\(^{2+}\) selective channel, which can be blocked by Lanthanum, an inhibitor of SOCs [Montell et al., 1985; Hardie and Mink, 1992]. The other two homologues, TRPL, which was originally isolated as a calmodulin (CaM) binding protein and showed to form a nonselective cation channel, and TRPy, which was shown to form heteromultimers with TRPL, were cloned afterwards [Philips et al., 1992; Reuss et al., 1997; Xu et al., 1997; 2000; Scott et al., 1997; 1998].

At least two functional aspects about TRP proteins have drawn intense interests. First, the activation of TRP is strictly dependent on PLC-mediated pathway since PLC-deficient mutant diminishes the light induced cation influx [Montell, 1998]. Second, a number of studies from the heterologous expression of TRP and TRPL have linked the expression of these channels with the functions of SOCs. Heterologous expression of TRP and TRPL in *Xenopus* oocytes, insect Sf9 cells, *Drosophila* S2 cells and HEK293T cells generated cationic conductance, either Ca\(^{2+}\) selective (TRP) or cation nonselective (TRPL). TRP and TRPL channels were activated upon depletion of Ca\(^{2+}\) from the internal stores using thapsigargin, an inhibitor of Ca\(^{2+}\)-ATPase on ER/SR [Vaca et al., 1994; Peterson et al., 1995; Xu et al., 1997; Yagodin et al., 1998]. Therefore, *Drosophila* phototransduction has been proposed to be a model system for the genetic dissection of SOCs, which are composed of TRP, TRPL and TRPy. It was postulated that very likely vertebrate homologues of the *Drosophila* TRP form channels mediating the store-operated and/or receptor-operated conductance.
1.2.2 Mammalian homologues of TRP

A number of TRP homologues (including different splicing variants and pseudogenes) have been cloned from mammalian species [Wes et al., 1995; Zhu et al., 1996; Harteneck et al., 1995; Ohki et al., 2000]. Based on the primary structure and results from the glycosylation scanning and mutagenesis studies, it is generally accepted that TRPs have six transmembrane segments (S1-S6) and a pore region located between S5 and S6. Both amino- and carboxyl- termini of TRPs reside at the cytoplasmic side of the plasma membrane [Vannier, et al., 1998]. The most conserved region, EWKFAR, located in the C-terminal vicinity of the S6 segment is called TRP domain. There are also conserved ankyrin-like repeats at the N-terminus and proline-rich motif at the C-terminus, both of which are suggestive of sites for protein-protein interactions. One or more CaM-binding domains have been found at the C-terminus, indicative of calcium/CaM-dependent regulation. By analogy to voltage-gated sodium and calcium channels, which have four tandem repeats of six transmembrane segments in a single polypeptide chain, it has been proposed that each TRP encodes a single subunit and must coassemble with other subunits to form functional channels, in the form of either homo- or heterotetramers. An important difference between TRP channels and VGCCs is that TRPs lack the critical positive charges in the S4 domain that act as voltage sensor in VGCCs. This is consistent with the fact that TRPs form SOCs gated by intracellular signals but not changes in membrane potentials. To date, seven canonical mammalian TRP homologues, TRPC1-7, have been found. Functional studies of heterologously expressed TRPC proteins have
shown that they are involved in Ca\textsuperscript{2+} entry from the extracellular environment after the activation of PLC or store depletion, similar to *Drosophila* TRP and TRPL [Wes et al., 1995; Zhu et al., 1996; Okada et al., 1998].

In addition to the TRPC channels, a large number of proteins with low homology to TRP have been claimed as TRP homologues according to structure characteristics. These include vanilloid receptor (VR1 or capsaicin receptor) and vanilloid receptor-like protein (VRL1), which are involved in the transduction of painful stimuli [Caterina et al., 1997, 1999]; osmotically activated channels [Strotmann et al., 2000]; Ca\textsuperscript{2+} transporters [Hoenderop et al., 1999; Peng et al., 1999]; novel channels notable for containing enzymatic domains in their C termini [Runnels et al., 2001]. Till now, there are approximately 20 different nonallelic mammalian TRP proteins. Searches of *Caenorhabditis elegans* genome database using sequence regions that are highly conserved among all TRP homologues identified 13 TRP-homologues [Wilson et al., 1994; Colbert et al., 1997]. A nomenclature system has been proposed for mammalian TRP homologues, which was agreed by most principle investigators in the TRP field (Fig. 1) [Montel et al., 2002]. The new nomenclature will minimize the confusion arisen from the different names used by different groups in publications.
Figure 1. Phylogenetic Tree of the TRP Superfamily. PAM, point accepted mutations. (Adapted from Cell vol.108 page 595-598)
1.2.3 The classification of TRP superfamily

The TRP superfamily has been classified into three families based on sequence homology (Fig. 1). The TRPV (‘V’ for vanilloid receptor) family contains five members: TRPV1, 2, 4, 5 and 6. Vanilloid receptors, TRPV1 and TRPV2, are heat-activated channels, which are also the best functionally characterized TRP channels. TRPV1 forms relatively Ca\(^{2+}\) selective ion channel with an outward rectification and Ca\(^{2+}\) dependent desensitization. Studies of TRPV1 knock-out mice showed that the protein was essential for transducing the nociceptive, inflammatory and hypothermic effects of vanilloid compounds [Caterina et al., 1997]. TRPV4 forms a channel responding to extracellular osmolarity changes instead of store depletion. TRPV5 and TRPV6 are highly Ca\(^{2+}\) selective channels present in epithelial cells of gut and kidney [Peng et al., 1999]. The TRPM (‘M’ for melastatin) family has eight members: TRPM1-8. They are the least known and the most novel proteins with very long sequences (around 1600 aa instead of ~900 aa as in the other two subfamilies), particularly in their N-termini. They are probably involved in cell growth, cell death and differentiation. The expression of TRPM1 (melastatin) correlates with cutaneous melanoma tumour progression. TRPM2 is a bifunctional protein, which forms a channel and confers ADP-ribose pyrophosphatase activity. The functions of TRPM3 and 4 are unknown. TRPM5 is associated with loss of heterozygosity in a variety of tumors. TRPM6 contains a putative α-kinase domain and probably forms a transmembrane protein. TRPM7, also known as TRP-PLIK (transient receptor potential-phospholipase interacting kinase), is the first member in this group to be shown as a functional channel [Runnels et al., 2001]. The channel activity is regulated by
intracellular ATP concentration and by its own C-terminal kinase, which is unique among ion channels in the TRP family. TRPM8, originally found to be upregulated in prostate cancer, was reported to form a cold/menthol-sensitive receptor in dorsal root ganglion [Mckemy et al., 2002; Peier et al., 2002]. TRPC (‘C’ for canonical) family has seven members. They are most closely related to Drosophila TRP and TRPL with identity scores ranging from 30% to 40%. Each of the TRPC proteins has been shown to form ion channels after heterologous expression. These channels are activated downstream of the stimulation of receptors that activate different isoforms of PLC. Some of the TRPC-formed channels have also been shown to be activated by store depletion (Fig. 1).

1.2.4 Characterization of TRPC family

Tissue distributions of TRPC1-7 have been determined by northern blotting, reverse transcriptase polymerase chain reaction (RT-PCR) and in situ hybridization (Table 1). These studies showed a very broad expression pattern of TRPC transcripts ranging from neuronal tissues like cerebellum and hippocampus to peripheral tissues like heart and kidney. Each TRPC has a distinct expression pattern [Hoffman et al., 2000]. Some are present in a wide range of tissues (e.g., TRPC1), whereas others are highly restricted (e.g., TRPC2, 3 and 5). TRPC3, 4 and 5 are almost exclusively or strongly expressed in the brain. TRPCs are also expressed in established cell lines. For example, TRPC3, 4 and 6 are expressed in HEK293 cells [Garcia et al., 1997]. TRPC1, 3, 4 and 5 are in bovine aortic endothelial cells whose levels are even regulated by different hormones [Chang et al., 1997]. It was shown that, β-estradiol significantly down-regulated TRPC4 while
Table 1 Tissue distribution of TRP channels (*MTN* multiple tissue northern blotting, *RT-PCR* reverse transcriptase polymerase chain reaction, *ISH* in situ hybridization).

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Species</th>
<th>Method</th>
<th>Tissue Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRPC1</td>
<td>Human</td>
<td>MTN</td>
<td>Heart, brain, testis, ovary, intestine</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>RT-PCR</td>
<td>Heart, brain, kidney, intestine, adrenal gland</td>
</tr>
<tr>
<td>TRPC2</td>
<td>Cattle</td>
<td>MTN</td>
<td>Testis, liver, spleen</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ISH</td>
<td>Late spermatogenic cells</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>MTN</td>
<td>Testis, cerebrum, cerebellum, heart</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>ISH</td>
<td>Vomeronasal organ sensory epithelium</td>
</tr>
<tr>
<td>TRPC3</td>
<td>Human</td>
<td>MTN</td>
<td>Brain</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>ISH</td>
<td>Cerebellar Purkinje cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MTN</td>
<td>Brain</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>RT-PCR</td>
<td>Cerebellum, midbrain, olfactory bulb, cortex</td>
</tr>
<tr>
<td>TRPC4</td>
<td>Cattle</td>
<td>MTN</td>
<td>Adrenal gland, testis, retina, heart, brain</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>MTN</td>
<td>Cerebrum, adrenal gland, cerebellum, ubiquitous</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ISH</td>
<td>Dentate gyrus granule cells, ca1 pyramidal neurons, cortex</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>RT-PCR</td>
<td>Olfactory bulb, hippocampus, cortex, cerebrum, nodose ganglion, testis, ovary, heart, lung</td>
</tr>
<tr>
<td>TRPC5</td>
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<td>Brain</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>RT-PCR</td>
<td>Brain, testis, kidney, uterus</td>
</tr>
<tr>
<td></td>
<td>Rabbit</td>
<td>MTN</td>
<td>Brain</td>
</tr>
<tr>
<td>TRPC6</td>
<td>Human</td>
<td>MTN</td>
<td>Lung, placenta, ovary, spleen, ubiquitous</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>MTN</td>
<td>Lung, brain</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>RT-PCR</td>
<td>Lung, cerebrum, ovary, ubiquitous, cerebral cortex, hippocampus, heart, kidney, lung, adrenal gland</td>
</tr>
<tr>
<td></td>
<td>ISH</td>
<td></td>
<td>Dentate gyrus granule cells, cerebral cortical neurons</td>
</tr>
<tr>
<td>TRPC7</td>
<td>Mouse</td>
<td>MTN</td>
<td>Heart, lung, eye, hindbrain, spleen, testis</td>
</tr>
<tr>
<td></td>
<td>ISH</td>
<td></td>
<td>Cerebellar Purkinje cells, olfactory bulb, hippocampus</td>
</tr>
</tbody>
</table>

trans-retinoic acid dramatically up-regulated TRPC5 in these cells. However, these hormones rendered little change in CCE, suggesting that the extent of a given TRPC channel's participation in CCE is not reflected in its transcript level. The functions of TRPC-formed channels have been studied using different systems. Most functional studies were carried out in heterologous expression system. Here, TRPC genes were overexpressed in either mammalian cell lines, such as HEK293 cells, or in *Xenopus* oocytes. The TRPC-expressing cells were treated with agents that either stimulate PLC or cause store depletion. There are two ways to activate PLC mediated signal transduction pathway: first, treating cells with receptor agonists, such as carbachol, will activate PLCβ through receptor binding and Gq protein coupling; second, treating cells with certain hormones and growth factors will activate PLCγ through receptor tyrosine kinases. To deplete internal calcium stores, special drugs have been used. These include thapsigargin (TG), which is an inhibitor of SR/ER Ca$^{2+}$-ATPase, and Ca$^{2+}$ ionophores such as ionomycin. Interestingly, it has been found that even the intermediate products of the PLC signal transduction pathway, for example, DAG and its derivatives can also activate TRPC channels. The activities of TRPC channels are measured by calcium imaging using cells loaded with fluorescence calcium indicators, such as fura-2 or indo-1. The TRPC-mediated membrane conductances are detected using electrophysiological methods, including whole cell patch-clamping and single channel recordings from cell-attached and inside-out patches. Increasing number of studies has now shown TRPC-like channel activities in native cells, such as neuronal cells or endothelial cells. Electrophysiological and pharmacological characteristics of the native channels have been compared with
those observed for TRPCs in heterologous expression system [Liu et al., 2000; Jungnickel et al., 2001]. TRPC family can be further divided into four groups: TRPC1, TRPC2, TRPC3,6,7 and TRPC4,5, according to sequence homology and functional similarity.

1.2.4.1 TRPC1

TRPC1 is the first cloned mammalian TRPC protein [Wes et al., 1995; Zhu et al., 1995]. There are at least four trpc1 gene variants reported in pancreatic cells [Sakura et al., 1997]. Human TRPC1 is 38% identical (62% similar) to Drosophila TRP and is expressed in the fetal brain and in the adult heart, brain, testis and ovaries at the highest levels. Lower levels of TRPC1 transcript have been found in almost all tissues.

TRPC1 appears to form SOC since functional expression of TRPC1 in CHO cells gave rise to a modest but significant enhancement of thapsigargin- and/or receptor-induced Ca\(^{2+}\) influx as measured by fura-2 [Zitt et al., 1996]. Furthermore, whole cell patch-clamp recordings after expression of TRPC1 in CHO cells showed an increase in IP\(_3\)- and thapsigargin- induced cation currents with similar permeability for Ca\(^{2+}\), Na\(^{+}\) and Cs\(^{+}\) [Zitt et al., 1997]. Studies in human salivary gland epithelial cells have demonstrated the presence of endogenous TRPC1 on the basal lateral membrane. Overexpression of TRPC1 increased channel activities alike SOC and enhanced salivary gland fluid secretion is TRPC1-dependent [Liu et al., 2000; Singh et al., 2001]. The correlation between SOC and the expression of TRPC1 has been established by the finding that a
decrease in the expression of endogenous TRPC1, by introducing antisense htrpc1 nucleotides into the cells, was associated with a decreased SOC activity while an increase in the expression of TRPC1, following stable transfection of the cells with htrpc1 cDNA, resulted in an increase in SOC channel activity. Additional evidence for the involvement of TRPC1 in SOC in native cells came from a study on bovine endothelial cells and human umbilical vein endothelial cells [Antoniotti et al., 2002; Groschner et al., 1998]. Anti-TRPC1 antibody revealed the presence of the protein only in the endothelial cell membrane compartment and the application of the antibody caused a partial but significant reduction of calcium entry induced by a biological angiogenic factor, bFGF [Antoniotti et al., 2002].

However, work from a different group showed that TRPC1 formed a non-selective cation channel that was constitutively active when expressed in Sf9 cells. The channel was insensitive to the depletion of internal calcium stores [Sinkins et al., 1998]. A study showed that in HEK293 cells, a 1-oleoyl-2-acetyl-sn-glycerol (OAG)-activated conductance was detectable in TRPC1-transfected cells only in a Ca\(^{2+}\)-free extracellular solution [Lintschinger et al., 2000]. Moreover, in HEK293 cells expressing TRPC1, when cells were stimulated with carbachol, an agonist that activates PLC\(\beta\), expression of TRPC1 did not result in measurable ion current [Strubing et al., 2001].
1.2.4.2 TRPC2

TRPC2 probably has the best known native function among all the TRPC channels. In human *trpc2* is a pseudogene since it contains premature stop codons and gaps when aligned with other TRPCs. However, *trpc2* cloned from mouse encodes a protein that forms store-depletion-activated CCE channel [Vannier et al., 1999]. TRPC2 participates in vomeronasal organ (VNO) sensory transduction in rat because it is highly expressed in the sensory microvilli of the VNO, which is thought to mediate social behaviors and neuroendocrine changes elicited by pheromone in rodent [Liman et al., 1999]. The sensory microvilli are the proposed sites of pheromone sensory transduction. The enrichment of TRPC2 at this location suggests a direct role of the channel in the pheromone-evoked response. The TRPC2 channel might represent the primary conductance activated by pheromone signals, or it could mediate a secondary amplification or modification of the sensory response. Recently, studies of TRPC2 knock out mice demonstrated that TRPC2 deficiency eliminated the sensory activation of VNO neurons by urine pheromones [Stowers et al., 2002]. TRPC2<sup>−/−</sup> male mice appeared unable to recognize the sexual identity, i.e., they failed to display the pheromone-evoked aggression toward male intruders that is normally seen in wild-type males, and they display courtship and mounting behavior indiscriminately toward both males and females. These data suggest that VNO activity and TRPC2 play critical roles in sex discrimination.

TRPC2 is also reported to be important in the fertilization process in mouse [Jungnickel et al., 2001]. The sperm acrosome reaction is a Ca<sup>2+</sup>-dependent secretory event that must be completed before fertilization. In mammals, exocytosis is triggered during gamete
contact by ZP3, a glycoprotein constituent of the egg's extracellular matrix, or zona pellucida (ZP). ZP3 activates G proteins and PLC and causes a transient Ca\(^{2+}\) influx into sperm through T-type Ca\(^{2+}\) channels. These early responses promote a second Ca\(^{2+}\)-entry pathway, thereby producing sustained increases in intracellular Ca\(^{2+}\) concentration that drive acrosome reactions. TRPC2 was shown to form the second Ca\(^{2+}\)-influx channel on the plasma membrane of sperms.

A bovine \textit{trpc2} homologue that contains only four transmembrane segments has been reported [Wissenbach et al., 1998]. Its expression is restricted in male reproduction tissues, suggesting that it might contribute to the formation of ion channels in sperm cells.

1.2.4.3 TRPC3/6/7 subfamily

TRPC3, TRPC6 and TRPC7 share 75\% amino acid identity and form non-selective cation channels [Zitt et al., 1997]. TRPC3 is highly enriched in rat neurons of the central nervous system and is only expressed around birth period in a pattern that correlates with the expression of neurotrophin receptor TrkB [Li et al., 1999]. Since TrkB is a neurotrophin receptor tyrosine kinase that activates PLC\(\gamma\)1, it seems that activation of TrkB by neurotrophin, BDNF (brain-derived neurotrophic factor), might lead to a TRPC3-mediated cation conductance that is dependent on PLC. It was confirmed \textit{in vivo} that TRPC3 contributes to a PLC-dependent, nonselective cation conductance in isolated pontine neurons from P3-P8 rats. In heterologous systems, TRPC3 is expressed on the plasma-membrane and is responsible for an agonist-dependent markedly enhanced Ca\(^{2+}\)
entry. Generally speaking, the overexpressed TRPC3 channel is not activated by store depletion with IP$_3$ or thapsigargin [Zhu et al., 1996; 1998; Boulay et al., 1997; Zitt et al., 1997; Hurst et al., 1998; McKay et al., 2000]. In CHO cells injected with a large amount of TRPC3 cDNA, the TRPC3-formed channel was spontaneously active and was further stimulated by an increase in intracellular [Ca$^{2+}$]. It was thus concluded that TRPC3 forms a Ca$^{2+}$ permeable channel that supports Ca$^{2+}$-induced Ca$^{2+}$ influx but not a SOC [Zitt et al., 1997].

However, the notion that TRPC3 did not form a SOC was not further substantiated. It appears that TRPC3 can form SOCs under certain conditions [Vazquez et al., 2001]. Expression of hTRPC3 in either wild-type or IP$_3$R-knockout DT40 avian B lymphocyte resulted in a substantially greater divalent cation influx after thapsigargin-induced store depletion, which was significantly larger in the wild-type cells than that in the IP$_3$R knockout cells. Therefore, TRPC3 is also a candidate for the store-operated nonselective cation channels in cells, which is activated in both IP$_3$-dependent and independent manners.

TRPC6 and TRPC7 are expressed in smooth muscle and heart cells at relatively high levels. There are at least three TRPC6 isoforms discovered from rat [Zhang and Safen, 2001]. When expressed in COS cells, rTRPC6 participated in the formation of Ca$^{2+}$ channels that were regulated by a G-protein-coupled receptor, but not by intracellular Ca$^{2+}$ stores. The three rTRPC6 isoforms showed different Ca$^{2+}$ or Ba$^{2+}$ influxes after
stimulation with carbachol or OAG. Based on the difference in sequence and in glycosylation patterns among the three rTRPC6 isoforms, it was concluded that the N terminus (3-56 amino acids) might be crucial for the activation of rTRPC6 by DAG and the segment located just downstream of the sixth transmembrane domain might be required for the processing of rTRPC6 protein [Zhang and Safen, 2001]. Studies in smooth muscle cells indicated that TRPC6 probably forms the native receptor-stimulated Ca\(^{2+}\)-permeable cation channels [Jung et al., 2001].

As TRPC3 and TRPC6, mouse TRPC7 form DAG activated and receptor stimulated channels in various native cells [Hoffman et al., 1999; Okada et al., 1999]. Recently, human TRPC7 (hTRPC7) was cloned from brain. It shares 98% homologous to mouse TRPC7 (mTRPC7) [Riccio et al., 2002]. hTRPC7 is widely expressed in tissues of the central nervous system, as well as some peripheral tissues such as pituitary gland and kidney. However, in contrast to mTRPC7, which is highly expressed in heart and lung, hTRPC7 was undetectable in these tissues. Expressing hTRPC7 cDNA in HEK293 cells resulted in a marked increase of Ca\(^{2+}\) influx with either carbachol or thapsigargin treatment. This increased Ca\(^{2+}\) entry was blocked by inhibitors of CCE such as La\(^{3+}\) and Gd\(^{3+}\). Furthermore, expression of antisense hTRPC7 construct into cells eliminated the augmented store operated Ca\(^{2+}\) entry by hTRPC7. All these functional data suggest that hTRPC7 forms a store-operated calcium channel distinctly different from mTRPC7, which enhances Ca\(^{2+}\) influx independently of store depletion.
1.2.4.4 TRPC4/5 subfamily

There are controversial reports on TRPC4 and TRPC5 about whether or not they form SOCs. Some laboratories reported that they were activated through PLC pathway by agonist binding while other groups showed that they were also activated by store depletion [Philipp et al., 1998; 2000; Okada et al., 1998; Schaeffer et al., 2000; Yamada et al., 2000]. Some group even could not detect activated TRPC4 channel activities under any given conditions [Mckay et al., 2000]. The difference probably came from the use of different expression systems (e.g., different cell lines), different sources of the TRPC4 or 5 genes (bovine or murine) or different splice variants of TRPC4. Two hTRPC4 splicing variants have been found and they react differently with IP$_3$R C-terminus, suggesting that the way hTRPC4-IP$_3$R complex is formed may be different for the two splicing variants of TRPC4 [Merry et al., 2001].

Native TRPC4 function was studied using bovine adrenal cortex cells which were found to express significant amount of TRPC4 [Philipp et al., 2000]. These cells displayed endogenous CRAC-like currents after IP$_3$- or thapsigargin-induced Ca$^{2+}$ store depletion. Transfection of these cells with TRPC4 antisense cDNA reduced both the endogenous CRAC-like current as well as the amount of endogenous TRPC4 protein. Therefore, TRPC4 forms at least a part of native CRAC-like channels in adrenal cells. TRPC4 knockout mouse is the first knockout model in the TRPC family. The elimination of TRPC4 reduced an I$_{CRAC}$-like store-operated current in endothelial cells [Freichei et al., 2001]. This work also revealed a physiological function of TRPC4 in the regulation of
vascular tone in mice and provided the strongest evidence so far for a store-dependent activation of mammalian TRP homologues.

Similar to TRPC4, TRPC5 can form homomeric cation channels that are activated by stimulation of PLC, without a significant response to store depletion [Okada et al., 1998; Schaeffer et al., 2000]. However, another study showed that rabbit TRPC5 could be activated by store depletion with thapsigargin [Phillipp et al., 1998]. Like TRPC4, there is no report showing that TRPC5 can be activated by DAG [Hofmann et al., 1999; Schaefer et al., 2000]. TRPC5 is predominantly expressed in vertebrate brain. The biophysical characteristics of TRPC5 activity, which is very important in distinguishing the TRPC5 channel in native neuronal preparation, has been established in HEK293 expressing system [Yamada et al., 2000].

1.2.4.5 TRPC forms SOCs through multimerization

Electrophysiological analyses have shown that TRPCs have characteristics similar to those reported for native SOCs including the CRAC channels. However, when expressed individually, no TRPC possesses the identical channel properties as the CRAC channel. ICRC, first recorded in rat basophilic leukemia (RBL) cells and Jurkat cells, has well-defined channel properties with very low single channel conductance, high calcium selectivity, inward current rectification and Ca2+-dependent inactivation [Hoth and Penner, 1992]. However, individually expressed TRPC channels typically have high single channel conductances (20-60 pS), are nonselective to cations and do not show
inward rectification. Therefore, it is unlikely that the CRAC channel is formed by a single
type the TRPC protein. On one hand, it is possible that only the cation nonselective SOCs
and/or receptor activated channels are formed by TRPC proteins. On the other hand, there
are several reasons to believe that TRPC could participate in the formation of Ca\(^{2+}\)
selective SOCs, including the CRAC channel, in the form of subunit of multimeric
channel complex:

First, the most convincing evidence is that, when antisense nucleotide sequences were
introduced to block TRPC gene expressions, either in overexpressed system or in native
cells not expressing exogenous TRPCs, significant reduction of SOC current or activity
was observed [Zhu et al., 1996; Liu et al., 1998; Wu et al., 2000]. In some cases,
complete elimination of SOC current was achieved after the transfection of a mixture of
plasmids containing antisense sequences for all TRPC clones [Zhu et al., 1996].

Native SOC activities were also reduced or eliminated when TRPC antibodies were
introduced or TRPC gene was knocked out. In TRPC4\(^{-/-}\) mice, a store-operated Ca\(^{2+}\)
current in vascular endothelium recorded in wild type mice is missing [Freichel et al.,
2001]. When anti-TRPC2 antibody was introduced to sperms, the acrosomal reactions
(with two steps of Ca\(^{2+}\) entry) did not proceed [Jungnickel et al., 2001]. Application of
TRPC1 antibody caused a partial but significant reduction of calcium entry induced by a
biological angiogenic factor, bFGF [Antoniotti et al., 2002].

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Second, the diversity of SOCs may be generated from a variety of coassembly of TRPC proteins. TRPCs form either homotetramers or heterotetramers, based on their structure characteristics. It is possible that the native SOCs are heterotetramers of specific sets of TRPCs. Examples of TRP multimerization have been shown in *Drosophila*, where coassembly of TRP and TRPL produced a novel store-operated, outward rectifying current distinct from the current owing to the expression of either TRP or TRPL alone [Gillo et al., 1996; Xu et al., 1997]. TRP forms channel with small outward currents whereas TRPL channel is constitutively active. Direct association between TRP and TRPL was demonstrated by co-immunoprecipitation (Co-IP) indicating that the TRP-TRPL-dependent current is mediated by a channel formed through heteromultimerization of the two subunits. Analysis of endogenous light sensitive channels in *Drosophila* photoreceptor cells has provided evidence supporting the hypothesis that the property of the wild-type conductance could be quantitatively accounted for by the combination of two components isolated from the *trp* and *trpl* mutant [Reuss et al., 1997].

Coassembly of mammalian TRPCs has also been reported. Coassembly of TRPC1 and TRPC3 proteins generated a constitutively active, DAG and Ca\(^{2+}\) sensitive cation channel in HEK293 cells. However, the expression of TRPC1 alone gave a DAG activated conductance that was only detectable under Ca\(^{2+}\)-free extracellular solution, while TRPC3 forms a DAG activated conductance detectable under physiological Ca\(^{2+}\) concentration (1.2 mM) [Lintschinger et al., 2000]. Recently, it was found that TRPC1 also coassembled with either TRPC4 or TRPC5 to form heteromultimeric cation channels.
in mammalian brain [Strubing et al., 2001]. The three TRPC proteins have overlapping
distribution in the hippocampus. When expressed in HEK293 cells, the channel formed
by TRPC1 alone did not carry significant current, while the one formed by TRPC5
carried a carbachol-stimulated inwardly rectifying current with single channel
conductance of 38 pS. A novel outwardly rectifying current was generated in cells
coeexpressing TRPC1 and TRPC5. While the combined nonselective cation channel was
still activated by the stimulation of Gq-coupled receptors, its single channel conductance
is much lower (5 pS). Both TRPC4 and TRPC5 were shown to coassemble with TRPC1
to form novel channels. Therefore, it is common that TRPCs coassemble to form
heteromultimeric channels. Based on the current hypothesis that four subunits are needed
to form a functional TRPC channel and the finding that several TRPC proteins are
colocalized in same tissues and cells, it can be predicted that with the presence of seven
TRPCs in mammals, the possible combinations to form heterotetramers are tremendous.
The discrepancies on the electrophysiological properties between native SOCs and
heterologously overexpressed TRPC channels probably came from the wrong subunit
compositions of the channels in the overexpression systems.

Obviously, trying to find a correct combination of TRPCs to reconstitute a native SOC is
a very difficult task. Functional characteristics of SOC and heterologously overexpressed
TRPC channels have been hindered by the lack of specific agonist for these channels. In
contrast to other well-defined ion channels such as VGCC, which have specific
pharmacological agents to distinguish them from other channels, there is no effective
agent that can specifically distinguish SOC from other channels. Comparison between native SOCs and TRPC channels can only be made through thorough electrophysiological characterization, which is also very difficult given that except for the CRAC channel in a limited number of cells, other SOCs have only been poorly characterized. Therefore, current efforts are still made on SOC reconstitution using various combinations of TRPC proteins.

1.3. Mechanisms for TRPC activation

The mechanisms on how SOCs are activated are still under debate. Regardless whether SOCs are formed of TRPCs or not, it remains a question how the information on the Ca\(^{2+}\) content in the ER lumen is transferred from internal Ca\(^{2+}\) stores (ER/SR) to the channels on the plasma membrane. Considerable attention has been focused on the signal that links the Ca\(^{2+}\) stores and the SOCs in recent years. Many hypotheses have been proposed and three major ones have been discussed widely. They are (1) soluble messenger hypothesis, (2) conformational coupling hypothesis and (3) secretion-like coupling hypothesis.

1.3.1 Soluble messenger hypothesis

The easiest way to explain the transfer of store depletion signal from intracellular stores to the plasma membrane is to assume the presence of a diffusible messenger [Putney, 1990]. The possible candidates would probably be one of the intermediate products involved in the PLC-mediated pathway. However, it turns out that none of them, PLC, DAG, PKC and IP\(_3\), is the universal activator of SOCs. It was then proposed that the activator should be a novel molecule. This idea was supported experimentally by the
work of Randriamampita and Tsien [Randriamampita and Tsien, 1993]. Extracts from store-depleted Jurkat T cells caused Ca\(^{2+}\) influx when applied to macrophages, astrocytoma cells, and fibroblasts. Further fractionated and purified extracts activated Ca\(^{2+}\) influx when injected into Xenopus oocytes [Kim et al., 1995]. When the extracts were applied to excised SOC-containing membrane patches, they also activated the channels on the membrane [Kim et al., 1996]. Therefore, there might be an activator of SOCs in the cell extracts from the activated T cells. The novel soluble mediator was therefore named CIF (Ca\(^{2+}\)-influx factor). CIF appears to have hydroxyls (or hydroxyl and amino groups) on adjacent carbons, a phosphate, and with molecular weight less than 500 Dalton [Randriamampita and Tsien, 1993].

The main problem of this proposal is that the molecular identity of CIF is still unknown. Another potential problem is that the channel activated by CIF is lanthanum insensitive, while the endogenous SOC channels are inhibited by lanthanum [Csutora et al., 1999].

1.3.2 Conformational coupling model

This model proposes that the communication between intracellular Ca\(^{2+}\) store and the plasma membrane Ca\(^{2+}\) influx channels involves a direct protein-protein interaction. The coupling between the intracellular Ca\(^{2+}\) release channels on the stores (IP\(_3\)Rs or RyRs) and SOCs located on the plasma membrane is analogous to excitation-contraction coupling between the L-type Ca\(^{2+}\) channel and the RyR in the skeletal muscle [Irvine, 1990]. In the skeletal muscle cells, the L-type Ca\(^{2+}\) channel senses the plasma membrane
depolarization and then activates the RyR on the SR. In turn through this direct protein-protein interactions, the RyR also regulates the gating of the L-type Ca\(^{2+}\) channel [Nakai et al., 1996].

Similarly, it has been shown that RyRs can also gate SOCs via conformational coupling [Kiselyov et al., 2000]. Ca\(^{2+}\) release from RyR-sensitive stores activated hTRPC3 in the hTRPC3 expressed HEK293 cells, and an endogenous SOC in human submandibular gland (HSG) cells. In cell attached as well as excised patches, hTRPC3 channel was activated by the stimulation of RyRs using caffeine. Association between RyRs and hTRPC3 was demonstrated by Co-IP with antibodies recognizing either RyRs or hTRPC3. Furthermore, the activation of hTRPC3 by caffeine was lost after extense wash of the cytoplasmic side of the excised patch by the perfusion buffer. The activity was restored by the addition of microsomal preparation containing high concentrations of native or recombinant RyRs.

IP\(_3\)Rs, the other type of Ca\(^{2+}\)-releasing channels of internal Ca\(^{2+}\) stores, have been shown to be involved in the activation of SOCs in many cell types and systems. Prevention of TRPC channel activation by an IP\(_3\)R inhibitor, 2-aminoethoxydiphenyl borate, indicated that IP\(_3\)Rs were essential for maintaining coupling between TRPCs and internal stores [Ma et al., 2000]. Electrophysiological evidence showing direct involvement of IP\(_3\)R on TRPC activation also came from TRPC3 expressing cells [Kiselyov et al., 1998]. The channel activities in excised membrane patches, which were restored by the addition of
IP$_3$ after a short period of perfusion, could only be restored by the addition of IP$_3$R and IP$_3$ together after prolonged perfusion. This indicated that IP$_3$Rs were required for TRPC3 channel activation after agonist binding. Co-IP experiments have demonstrated the association between IP$_3$R and TRPC1, 3 and 6. Glutathione S-transferase (GST) pull-down experiments showed direct interaction between IP$_3$R and TRPC3 for the N-terminus of IP$_3$R and the C-terminus of TRPC3 [Boulay et al., 1999]. To test the effects of the interaction domains on Ca$^{2+}$ entry activated either by receptor agonist or by store depletion, the IP$_3$R-binding region of TRPC3 and TRPC3-binding region of IP$_3$R were introduced into HEK293T cells. Measurement of Ca$^{2+}$ influx in these cells by intracellular Ca$^{2+}$ imaging showed that overexpression of the TRPC-IP$_3$R interaction domains interfered with the agonist activated and store-operated Ca$^{2+}$ entry [Boulay, et al., 1999]. These indicate that IP$_3$Rs, in addition to mediating Ca$^{2+}$ release from internal stores, can also directly activate Ca$^{2+}$ influx through interaction with TRPC channels on the plasma membrane in response to either increasing cytosolic IP$_3$ or decreasing luminal Ca$^{2+}$ concentration. All three subtypes of IP$_3$Rs have been shown by GST pull down experiments to directly interact with all TRPC proteins, demonstrating that direct coupling between TRPCs and IP$_3$Rs may be an important and ubiquitous gating mechanism for TRPC channels [Lockwich et al., 2000; Rosado et al., 2000; Tang et al., 2001].

The understanding of conformational coupling mechanism was further enhanced by the finding that a CaM-binding site overlaps with the IP$_3$R-binding domain on the C-terminus
of TRPC3 [Zhang et al., 2001]. The common binding site is called CIRB (CaM and IP₃ binding) site. Direct competition between IP₃ and Ca²⁺/CaM for CIRB site on TRPC3 was demonstrated in in vitro binding assays, suggesting that CaM has a regulatory effect on the activation of TRPC3 by IP₃Rs. Functional data using electrophysiological recording on excised patches showed that TRPC3, which has an otherwise high channel activity that is suppressed by Ca²⁺/CaM under resting conditions, can be activated by displacing Ca²⁺/CaM from the common binding site with IP₃R, or by either removing Ca²⁺/CaM using a high affinity Ca²⁺/CaM binding peptide or inactivating Ca²⁺/CaM with a CaM antagonist. It was thus concluded that the displacement of CaM from CIRB site by activated IP₃Rs is an important step for TRPC3 activation. CaM also binds to the Drosophila light-sensitive ion channels, TRP and TRPL [Chevesich et al., 1997; Trost et al., 1999]. Equivalent CIRB sites have been shown for all TRPC proteins and additional CaM-binding domains have been found at the C-terminus of TRPC1, 2, 4 and 5 [Tang et al., 2001]. Channel regulation involving competitive association of regulatory protein with CaM on a common binding site has been shown for olfactory cyclic nucleotide-gated channel and the N-methyl-D-aspartate (NMDA) glutamate receptor [Varnum et al., 1997; Krupp et al., 1999]. Ca²⁺ and CaM have been reported to be involved in regulating native SOCs as well. Ca²⁺-dependent inactivation has been documented for SOCs in lymphocytes, pancreatic acinar cells and submandibular gland cells [Zweifach et al., 1995; Vaca et al., 1996; Louzao et al., 1996; Liu et al., 1998]. Therefore, the involvement of Ca²⁺/CaM in conformational coupling model is probably a ubiquitous mechanism for
the activation of TRPC-based channels, which is likely applicable to native receptor activated channels as well as SOCs.

The effect of CaM is Ca^{2+} concentration dependent. At low Ca^{2+} concentration, CaM does not significantly compete with IP_{3}R for binding to the CIRB site, whereas at higher Ca^{2+} concentrations, CaM causes a Ca^{2+} dose-dependent inhibition of interaction between IP_{3}R and TRPCs [Zhang et al., 2001]. In addition to TRPC3, all other TRPC members contain the common binding site for CaM and IP_{3}R S [Tang, et al., 2001]. This scenario raises the possibility of a common mechanism of TRPC channel regulation that involves the control by Ca^{2+} concentration in the immediate vicinity of the channel.

Even though a large body of evidence favors the conformational coupling of SOCs via interaction with RyRs and IP_{3}Rs, there are other experimental data that disagree with the idea. These mainly came from the studies of a DT40 pre-B-lymphocyte cell line in which all three IP_{3}R subtypes were eliminated by targeted gene disruption [Sugawara et al., 1997]. Although these cells do not respond to PLC-coupled agonist and intracellular application of IP_{3}, they show normal CCE in response to thapsigargin [Broad et al., 2001]. The ability of DAG to activate SOCs in the IP_{3}Rs knockout DT40 cells transfected with TRPC3 suggests that there at least exists another mechanism to activate SOCs independent of RyRs and IP_{3}Rs. Kiselyov et al. argued that the effect of IP_{3}R mutation in the DT40 cells may be incomplete in respect to conformational coupling because the sites of gene disruption on IP_{3}Rs were very close to the C-termini, which could result in the
formation of truncated IP₃ Rs [Broad et al., 2001]. The truncated IP₃ Rs could still interact with TRPCs and have the ability to regulate TRPC, even though they would not form functional IP₃-gated Ca²⁺-releasing channels on the internal Ca²⁺ stores. However, later studies from Putney’s group confirmed that the IP₃ R knockout DT40 cells did not express significant amount of even the truncated IP₃-binding proteins [Broad et al., 2001].

Neither wild type nor the IP₃ R-knockout DT40 cells responded to caffeine, which suggests that RyRs do not play a significant role in SOC activation in these cells [Broad et al., 2001]. Nevertheless, there was a report showing that RyR₁/³ proteins were detected in DT40 cells and they were activated by cADP-ribose. The activation of RyRs facilitated the activation of I₁CraC, which suggests that RyRs efficiently gate I₁CraC in DT40 cells and explain the persistence of I₁CraC gating by internal stores in the absence of IP₃ Rs [Kiselyov et al., 2001]. The controversial conclusions obtained by the two groups came from the distinct effect of RyR activators on cells from different species. Caffeine is an effective activator of mammalian RyRs, which probably has no effect on avian RyRs.

1.3.3 Secretion-like coupling model

This mechanism suggests that SOCs are activated through a secretion-like insertion of channel-containing vesicles into the plasma membrane by vesicle fusion in response to store depletion. Plasma membrane proteins move from the ER through Golgi apparatus to the cell surface. There are two types of secretion: one is constitutive secretion and the other is regulated secretion. In the constitutive secretion process, proteins, such as
collagen secreted by fibroblasts and serum proteins by hepatocytes, are sorted from the trans-Golgi network into transport vesicles, which immediately move to and fuse with the plasma membrane and release their contents by exocytosis. In certain cells, the secretion of a specific set of proteins is not continuous. These proteins are sorted in the trans-Golgi network into secretory vesicles, which are stored inside the cell waiting for a stimulus for exocytosis, such as neural or hormonal stimuli. This process is called regulated secretion. A rise in the cytosolic Ca\(^{2+}\) concentration induced by binding of hormone to its receptor is required to trigger fusion of the secretory-vesicle membrane with the plasma membrane in a GTP-dependent manner.

Secretion-like coupling model was originally brought up when a GTP-dependent step was found to be required to activate SOCs [Fasolato et al., 1993]. It was reported that, the activation of plasma membrane Ca\(^{2+}\) currents induced by the depletion of Ca\(^{2+}\) stores required a diffusible cytosolic factor and was blocked by guanosine 5'-3-O-(thio) triphosphate (GTP\(\gamma\)S) and guanyl-5'-yl imidodiphosphate, non-hydrolysable analogs of GTP. Yao et al. [1993] presented molecular evidence in favor of this idea using an electrophysiological approach. Depletion of Ca\(^{2+}\) stores in \textit{Xenopus} oocytes activated Ca\(^{2+}\) entry through SOCs across the plasma membrane. The activated current, known as \(I_{\text{soc}}\), was measured by electrophysiological recording from cell-attached and inside-out patches. If cell-attached patches were formed before store depletion, \(I_{\text{soc}}\) could not be activated inside the patches. The tight seal of the patch inhibited activation of new \(I_{\text{soc}}\) in the patch. Maintenance of such \(I_{\text{soc}}\) did not require presence of cytosolic substances.
These results suggest that activation of $I_{soc}$ is rather localized and unlikely to result from diffusion of an activator molecule. To explain these results, it was proposed that channels were incorporated into the plasma membrane upon store depletion via exocytosis. In the preformed patches, the number of channels within the patched membrane was fixed because the tight seal prevented the insertion of new channels to the patched membrane. Without the patch, the insertion of new channels occurred normally so that when new patches were made after cells were activated with IP$_3$ or store depletion, more channel activity was detected.

The involvement of the secretion-like coupling mechanism in the activation of SOC was tested using two kinds of agents: *Clostridium botulinum* C3 transferase and botulinum neurotoxins (BoNTs). C3 transferase specifically inactivates Rho through ADP ribosylation of Rho at Asn-41, which has been shown to increase insertion of the insulin-sensitive glucose transporter into the plasma membrane in 3T3-L1 adipocytes [Van den Berghe et al., 1996]. BoNTs are a group of zinc endoproteases produced by bacteria of the genus *Clostridium* and they display specific activity for a group of protein components in the exocytotic apparatus: a vesicle-associated membrane protein (VAMP or synaptobrevin), and two plasma membrane-attached proteins, SNAP-25 and syntaxin. BoNTs are widely used to block regulated exocytosis in secretory cells. Expression of excess Rho A or injection of C3 transferase, which either up- or down-regulates Rho A, decreased or increased the amplitude of $I_{soc}$, respectively. Rho A is known to regulate many cell events, including cytoskeletal rearrangement and membrane trafficking.
Because Rho A may affect both constitutive and regulated membrane trafficking, it was only concluded that trafficking was important for modulating capacitative Ca\textsuperscript{2+} entry. Evidence that regulated exocytosis may be important for the activation of $I_{\text{soc}}$ came from the finding that BoNT inhibited 50\% and dominant negative mutant of SNAP-25 completely inhibited $I_{\text{soc}}$.

Since cytoskeletal rearrangement is necessary for the interaction of secretory vesicle with plasma membrane, studying the effect of cytoskeleton distortion on SOC activation would shed some light on if a secretion-like coupling process is involved in the activation of SOCs. It was demonstrated that actin-mediated trafficking was important for the activation of SOC in smooth muscle cells and TRPC3 expressed in HEK293 cells since channel activation was inhibited by actin filament condensation induced by a phosphatase inhibitor, calyculin A [Patterson et al., 1999; Ma et al., 2000]. Since the targets of calyculin A and Rho are thought to be actin-binding proteins of the ezrin/radixin/moesin (ERM) family, these data also indicate that ERM proteins are involved in activating SOCs.

Some problems remain for the secretion-like coupling model. Even though the hypothesis may explain one of the steps involved in the activation of SOCs, it does not provide any information on how the activation is coupled to depleted Ca\textsuperscript{2+} stores. Furthermore, cytoplasmic Ca\textsuperscript{2+} concentration is very important to trigger the secretion process. However, SOCs can be activated strongly when intracellular Ca\textsuperscript{2+} concentration is
buffered to very low levels. The reduction of $[Ca^{2+}]_i$ itself would activate channels [Krause et al., 1999; Braun et al., 2000]. There is evidence that the fusion between secretory vesicle and plasma membrane is not necessary for channel activation [Patterson et al., 2000]. There are also reports suggesting that channels are present on the plasma membrane prior to activation because SOCs could be activated in inside-out patches following the excision from cells [Zubov et al., 1999; Braun et al., 2001].

1.3.4 Reconciliation of different hypotheses

It is possible that multiple mechanisms regulate the activation of TRPC. More than one mechanism can exist in the same activation process. For example, CIF and conformational coupling could act together to cause the activation of SOCs or TRPC channels, i.e., the activated cells first produce the CIF and then CIF facilitates the interaction between IP$_3$R and TRPCs. Similar cooperation may also be the case for conformational and secretion-like coupling mechanism. Combination of the two may help moving the vesicle-bound TRPC from the cytoplasm to the plasma membrane. Alternatively, conformational coupling regulates the activation status of the membrane channels while secretion-like coupling brings more channels to the plasma membrane, thus making them available for the activation by the former mechanism.

It is also possible that some models remain a suitable hypothesis for certain channel types but may not be necessarily applicable in all cases. For example, it is possible that conformational coupling is only involved in the activation of specific types of SOCs,
such as those found in endothelial cells, but not in other channels, such as the CRAC channel in blood cells. Other unknown mechanisms may also exist. It has been shown that SOCs and TRPCs activated through different means, either dependent or independent of conformational coupling via IP$_3$R, were all inhibited by a common pharmacological agent [van Rossum et al., 2000], suggesting that there is a general mechanism underlying the activation of all SOCs.

1.4. Cytoskeleton and intracellular scaffold proteins are involved in the activation of TRPC channels

Increasing evidence has indicated that secretion-like exocytotic process is involved even though it is probably not the sole mechanism for the activation of TRPC channels. As introduced above, many important proteins involved in the secretion, such as Rho A and SNAP25, play critical roles for channel activation. Intracellular structural proteins, such as cytoskeletal proteins and ERM proteins, are also involved in the process of channel activation. It is very common that intracellular proteins, especially PDZ (FSD-95/Dlg/ZO-1-like) domain-containing scaffold proteins, participate in the regulation of channel activities, both in neuronal tissues and in peripheral tissues.

1.4.1 Involvement of intracellular PDZ domain-containing proteins in channel regulation

Channels in neuronal tissues are regulated by intracellular PDZ domain-containing proteins and cytoskeletal proteins. For example, AMPA receptors are ligand-gated cation
channels in neuronal tissues, such as hippocampus and cerebellum. It has been shown that the activation of AMPA receptor is not dependent on the regulation of existing channels on the plasma membrane. Instead, it is dependent on the insertion of more AMPA receptors to the plasma membrane after stimulation [Hirai et al., 2001; Ehlers, M.D., 2000; Li et al., 1999; Lu et al., 2001; Luscher et al., 1999]. Accumulating experimental data have indicated that this process requires post-synaptic density proteins: a set of PDZ domain-containing proteins, such as glutamate receptor interacting protein (GRIP), AMPA receptor binding protein (ABP), protein interacting with C-kinase 1 (PICK1), and cytoskeleton-associated proteins such as yotiao, all of which are associated with AMPA receptors [Lin et al., 1998; Srivastava et al., 1999; Dong et al., 1999]. N-ethylmaleimide-sensitive fusion protein (NSF), an ATPase involved in membrane fusion during exocytosis, is also reported to interact with AMPA receptors [Osten et al., 1999]. NSF probably regulates the membrane insertion, stabilization and the functional expression of AMPA receptors [Luthi et al., 1999]. The activation of AMPA receptors, in which more AMPA receptor proteins are inserted to the plasma membrane, was shown to be a secretion process because the secretory proteins, such as SNAP, GTP-dependent dynamin and SNARE proteins, are involved [Lu et al., 2000].

It is also common for channels in non-excitable cells to be regulated by intracellular scaffold proteins. One example came from a cAMP-activated chloride channel, cystic fibrosis transmembrane conductance regulator (CFTR). CFTR is polarized to the apical plasma membrane in epithelial cells. Mutation of CFTR causes a common genetic disease
cystic fibrosis. Even though there is not direct evidence showing that the secretion process actually happens during the activation of this channel, it has been proposed that cAMP stimulates trafficking of CFTR from an intracellular pool to the plasma membrane and decreases endocytic retrieval of CFTR from the plasma membrane [Morris et al., 1998]. C-terminus of CFTR comprises a PDZ-interacting domain that is required for the polarization of CFTR to the apical membrane in human airway and kidney epithelial cells [Moyer et al., 1999]. One intracellular interacting protein, CAP70, has been identified as CFTR-binding protein. CAP70 is a four-PDZ domain-containing protein which is also concentrated on the apical surfaces [Wang et al., 2000]. CAP70 potentates CFTR chloride channel activities by linking at least two CFTR molecules together through the two of its PDZ domains. Therefore, the CFTR channel can be switched to a more active conducting state via a modification of intermolecular CFTR-CFTR contact that is enhanced by a multi-PDZ domain protein.

The regulation by intracellular protein is not only restricted to targeting and insertion of channel proteins onto the plasma membrane. It is also involved in the sorting of membrane proteins in the endocytic pathways. β2-adrenergic receptor is a seven-transmembrane-domain receptors, having an extracellular amino terminus and an intracellular carboxyl terminus. An intracellular PDZ domain-containing protein, Na+/H+ exchanger regulatory factor (NHERF), has been reported to form a direct agonist-promoted association with the β2-adrenergic receptor [Hall et al., 1998]. NHERF binds to the β2-adrenergic receptor by means of a PDZ domain-mediated interaction with the last
few residues on the carboxyl terminus of the receptor. Agonist-activated \( \beta_2 \)-adrenergic receptor undergoes internalization and then sorting between recycling endosomes and lysosomes. The association of \( \beta_2 \)-adrenergic receptor and NHERF controls this sorting operation since disruption of the interaction between them caused missorting of endocytosed receptor [Cao et al., 1999]. PDZ domain-containing protein mediated sorting mechanism may specifically control the membrane trafficking of certain signaling molecules, such as \( \beta_2 \)-adrenergic receptor, which is in contrast to the constitutive recycling of many other membrane proteins, transferrin receptor as one of the examples.

1.4.2 NHERF — a multifunctional two-PDZ domain-containing protein

NHERF is also known as ERM-binding phosphoprotein of 50 kDa (EBP-50) since the carboxyl terminal region of NHERF binds to amino-terminal domains of ERM proteins [Reczek et al., 1997; 1998]. NHERF is a physiologically relevant ERM-binding protein because it colocalizes with actin and ezrin and it was Co-IPed with ezrin. The ERM family of proteins, in which ezrin is the best studied member, are important for assembly and stabilization of specialized plasma membrane domains like microvilli and membrane ruffles [Bretscher et al., 1997]. The ERM proteins are reported to provide linkage between integral membrane proteins and cortical actin cytoskeleton, with the N-terminal halves associating with integral membrane proteins, either directly or indirectly through adapter molecules like NHERF, and their C-terminal halves associating with F-actin. However, ERM proteins normally exist in a dormant state with both membrane- and cytoskeletal- association sites masked through an intramolecular interaction between N-
and C-terminal domains. Dormant ERM proteins can be activated by C-terminal phosphorylation to expose both F-actin and NHERF binding sites [Simons et al., 1998]. The association with other ERM protein binding partners, including the regulatory subunit of protein kinase A (PKA) and rho-GDI, implies that ERM proteins are integral components of these signal pathways.

NHERF has a general function on regulating membrane proteins since it can interact with a group of membrane proteins such as receptors and ion transporters including CFTR, sodium-hydrogen exchanger (NHE3), platelet-derived growth factor receptor (PDGFR) and the P2Y1 purinergic receptor in addition to the β2-adrenergic receptor. These interactions have been implicated in sequestering interactive sets of proteins into common microdomains and regulating the activity and trafficking of interacting proteins [Shenolikar et al., 2001]. NHERF was originally isolated as a necessary cofactor in cAMP-associated inhibition of the renal brush border NHE3 [Weinman et al., 1995; Yun et al., 1997]. It was proposed and later confirmed that NHERF, ezrin and PKA from a multiprotein signaling complex linking NHE3 to the actin cytoskeleton to facilitate the phosphorylation and downregulation of NHE3 [Kurashima et al., 1999; Weinman et al., 2000a; 2000b; 2001]. NHERF contains two PDZ domains. The ability to self-associate through PDZ domains provides a mechanism for NHERF to expand its capacity on forming multiprotein complex [Fouassier et al., 2000]. Involvement of NHERF in a broad array of biological systems makes it distinct from its regulation in renal transporters. For example, the relationship between NHERF and CFTR has gained increasing interest.
CFTR contains a C-terminal NHERF interaction consensus sequence and binds to NHERF with high affinity [Wang et al., 1998; Sun et al., 2000]. Either of the two PDZ domains of NHERF bound to CFTR and this binding increased the open probability of the CFTR channel [Raghuram et al., 2001]. NHERF may also function as a membrane retention signal for CFTR [Short et al., 1998; Moyer et al., 1999]. The association between NHERF and YAP65 (Yes-associated protein) is also important for the proper localization of YAP65 and its concentration at the apical membrane in airway epithelia [Mohler et al., 1999]. Recently it has been reported that NHERF interacts with PDGFR [Hall, et al., 1998; Maudsley et al., 2000]. PDGFR is a receptor tyrosine kinase that mediates the mitogenic effects of PDGF by binding to and/or phosphorylating a variety of intracellular signaling proteins upon PDGFR dimerization. NHERF binds to PDGFR with high affinity through the PDZ domain and potentiates PDGFR autophosphorylation and Erk (extracellular signal-regulated kinase) activation inside cells. Same as the NHERF-NHE3 association, linkage of NHERF and PDGFR facilitates formation of a cytoskeleton-associated signaling complex that activates downstream signals.

NHERF-2, a close relative of NHERF, plays significant role in the regulation of mammalian PLCβ isoforms. NHERF-2 also contains two PDZ domains and shares 52% identity with NHERF [Yun et al., 1998]. Yeast two-hybrid system and Co-IP experiments showed that NHERF-2 specifically interacts with PLCβ3 [Hwang et al., 2000]. NHERF2 also unregulated PLCβ3 activated with the stimulation of a muscrinic receptor since wild type NHERF-2 potentiated carbachol (CCh)-stimulated PLCβ3 activity in COS and HeLa
cells while a mutant NHERF-2 without the ability to interact with the PLC had no such effect. The oligomerization of NHERF and NHERF-2 may also facilitate NHERF-mediated formation of cellular signaling complexes [Lau et al., 2001].

1.4.3 Transducisome formation through a scaffolding protein facilitates signal transduction in Drosophila eyes

TRP proteins in Drosophila have also been shown to be linked with an intracellular scaffold protein in a signaling complex. During the visual response that finally activates the TRP channels, light stimulates rhodopsin, which activates an eye-specific Gαq. Activation of Gαq triggers NORPA (no-receptor-potential A), a phospholipase C-β, which catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) to generate IP$_3$ and DAG. The final result of this visual signaling is the opening of cation channels, TRP and/or TRPL, leading to depolarization of photoreceptors. TRP was found to interact with a photoreceptor-specific protein containing five PDZ domains named INAD (inactivation-no-afterpotential D) [Hardie, 2001]. The disruption of the interaction between INAD and TRP resulted in an abnormal light response, indicating that INAD is a regulatory subunit of TRP channels [Huber, et al., 1996]. The finding that TRP lost its spatially restricted compartment in rhabdomere in the $inaD$ mutant suggest that INAD is required $in$ vivo for anchoring the ion channel to normal subcellular site [Chevesich et al., 1997]. Later, NORPA (no-receptor-potential A) and eye-specific PKC were all found to interact with INAD and the associations among all these proteins were found to be
essential for controlled activation and deactivation of *Drosophila* phototransduction [Shieh et al., 1997; Adamski, et al., 1998].

The INAD complex is a supramolecule that includes a minimum of seven proteins: a receptor (rhodopsin), an effector (PLC), regulators (PKC and CaM), ion channels (TRP and TRPL) and scaffolding protein INAD [Xu et al., 1998]. The supramolecular complex is called signal transducisome. The assembly of the massive complex is facilitated by homomultimerization of INAD itself. Among them, TRP and the PDZ protein, INAD, form the core complex required for retention of the signal complex at the rhabdomere of the *Drosophila* photoreceptor cells [Li et al., 2000]. INAD probably functions to preassemble the transducisome helping to ensure that the transduction complex has the appropriate composition and are placed in the proper location [Tsunoda et al., 2001]. Nearly every protein involved in phototransduction is a component of the transducisome. Therefore, the linkage of proteins involved in *Drosophila* phototransduction to INAD provides an explanation for the highly efficient signal transduction. Instead of random stochastic collision among the molecules, signaling through G-protein-coupled cascades is comprised of components that are physically coupled together.

In addition, INAD was shown to bind directly to NINAC (neither inactivation nor 'afterpotential C) myosin III, which in turn links the complex to the cytoskeletal protein, actin [Wes, et al., 1999]. Disruption of the association between NINAC and INAD delayed termination of the photoreceptor response. Therefore, through binding to the
scaffold protein, the cytoskeletal protein and myosin also play roles in rapid deactivation of the photoresponses in *Drosophila*.

**1.4.4 TRPC channel activity is probably regulated by intracellular scaffold proteins and cytoskeleton**

The PLC-mediated pathway in mammalian cells is very similar to that in *Drosophila* photoreceptors. It is reasonable to predict that there must be mammalian PDZ domain-containing scaffold proteins to tether all or some of the signaling components together, in a manner similar to INAD.

A large number of PDZ domain-containing proteins have been found in the recent years. Growing evidence suggest that PDZ domains are important protein-protein interaction sites for clustering and organization of signaling molecules, particularly ion transporting proteins [Ranganathan and Ross, 1997]. Although several multi-PDZ domain-containing proteins distantly related to INAD have been cloned from human and rodents [Philipp et al., 1997; Ullmer et al., 1998; Kurschner et al., 1998], none has been shown to interact with mammalian TRPC. On the other hand, the last several C-terminal residues of a target protein often determine to which PDZ domain the protein associates. Since not only the C-terminal sequences of mammalian TRPCs are very different from their *Drosophila* counterpart but also they are very diverse among themselves, each TRPC may bind to a different PDZ domain. The PDZ domain-containing proteins that bind to the mammalian TRPCs may be quite different from that of INAD.
NHERF-2 is the only PDZ protein that has been shown to interact with PLCβ isoforms in mammalian cells. The binding partners of NHERF were identified after screening of random peptide library. Peptides terminating with the amino acid sequence “TRL” bound with high affinity to the first PDZ domain of NHERF [Wang et al., 1998]. TRPC4 and TRPC5 contain a carboxyl terminal motif (VTTRL) not present in other TRPCs, which is consistent with the conserved NHERF-binding motif. When clustered with NHERF, the channels will be parts of multimolecular signaling complexes since NHERF also bind to many ion transporters and receptors. Furthermore, during signaling, NHERF may play a role in dynamically controlling the redistribution of associated proteins, such as channels and receptors or intracellular messengers [Moyer et al., 1999; Mohler et al., 1999; Shenolikar et al., 2001]. Elucidating the functional role of the protein-protein interactions within these signaling complexes will be crucial and helpful for the understanding of TRPC channel regulation.

1.5. Aims of the project and summary of findings

Aims: First, since PDZ domain-containing proteins are involved in channel regulation and NHERF has been shown to interact with proteins possessing a C-terminal binding motif that is also present in TRPC4/5, I wanted to determine if NHERF is associated with TRPC4/5 and whether it is involved in the formation of a signal complex similar to INAD. Second, I tested the secretion-like coupling model using TRPC4 as an example. Since cytoskeleton and scaffold proteins play important roles in vesicle secretion, I
wanted to verify if TRPC4 is physically connected with those proteins and to test whether the connection has any effect on channel activation.

Summary of findings: I show that indeed, TRPC4 and TRPC5 bind to the first PDZ domain of NHERF. Moreover, the same PDZ domain also binds to the C-termini of PLCβ1 and PLCβ2, indicating that similar to INAD in Drosophila eyes, NHERF is capable of bringing together the signaling molecules involved in the PLC-mediated pathway in mammalian cells. I demonstrated this by Co-IP of PLCβ1 from mouse brain by anti-TRPC4 and anti-NHERF antibodies. In addition, I show by confocal fluorescence microscopy that upon stimulation with thapsigargin, TRPC4 undergoes redistribution on the plasma membrane, supporting the idea that membrane fusion and/or actin redistribution are involved in SOC activation. I also provide physical evidence supporting the secretion-like coupling model using TRPC4 as a representative SOC channel. The linkage between TRPC4 and actin was demonstrated to be NHERF and ERMs. I show that their association can be increased after either PLC activation with a receptor agonist or store depletion with thapsigargin. At the same time, the amount of TRPC4 protein on the plasma membrane is also increased following PLC stimulation, consistent with the idea that insertion of new channels on the cell surface is required for channel activation.
CHAPTER 2

METHODS

2.1. Mouse and rat strains

Mice in FVB/N background were used for detection of tissue distribution of TRPC4 in western blotting, immunoprecipitation and immunohistochemical assays. Sprague-Dawley rats were used for the isolation of rat TRPC4 by RT-PCR.

2.2. Cell Culture

HEK293 cells were maintained under subconfluent conditions at 5% CO₂ in DMEM (Dulbecco’s minimal essential medium) containing 4.5 mg/ml glucose, 10% heat-inactivated fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin at 37°C. Cells were subcultured twice weekly.

2.2.1 Transient transfection

For transient expression of DNA constructs, \(1 \times 10^6\) cells of the background cell line, either HEK293 cells or established stable cell lines, were seeded on a 10 cm dish for 20 hrs. The cells were transfected with 4 μg of plasmid containing the cDNA of the gene of
interest using Lipofectamine Plus Reagents (Invitrogen-Life tech.) following manufacturer’s protocol and examined 24 hrs later.

2.2.2 Stable cell line selection

$2.8 \times 10^6$ cells were plated on a 6-well plate 20 hrs prior to transfection. 1 µg of interested plasmid was transfected into the cells using Lipofectamine Plus Reagents following manufacturer’s protocol. After 24 hours, cells were harvested, diluted in medium supplemented with 400 µg/ml G418, and transferred to 96-well plates in three serial dilutions of 1:20. G418-containing medium was exchanged every 3 to 4 days. After a month, G418-resistant transformants that appeared to arise from a single colony were expanded into 12-well plates. Clonal cell lines expressing desired proteins were identified by western blotting. Cells from the stable cell lines were subcultured twice weekly and maintained in medium supplemented with 400 µg/ml G418.

2.3 DNA constructs and stable cell lines

2.3.1 Constructs provided by Dr. Michael Xi Zhu and others

A. Wild type TRPC4α

Wild type full-length TRPC4α (Fig. 2) in pcDNA3 vector (Invitrogen) was transfected into HEK293 cells and G418-resistant stable clones were selected. One of the stable cell lines, A172-60, was used for this study.

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Figure 2. TRPC4 constructs expressed in HEK 293 cells. The constructs and corresponding stable cell lines were made according to description in Methods.
B. TRPC4-HAc

cDNAs encoding TRPC4α with the primary antigenic epitope YPYDVPDYA of the *Haemophilus influenza* virus hemagglutinin antigen (HA) at its C terminus was constructed by introducing into pIRESneo (Clontech) carrying the TRPC4 in-frame to the nucleotide sequence 5'-TAC CCG TAC GAT GTT CCT GAT TAC GCG-3' immediately before the TGA stop codon (Fig. 2). I transfected the resulting plasmid into HEK293 cells and the stable cell lines expressing TRPC4-HAc were established as A727. One of them, A727-1, was used for this study.

C. TRPC4-HAe

This construct was provided by Dr. Jisen Tang. cDNAs encoding TRPC4α carrying the HA epitope at the second extracellular loop between the two transmembrane domains (S3 and S4) immediately after the TAC (Y459) were constructed in pIRESneo (Fig. 2). The nucleotide composition was confirmed by sequencing. I transfected the resulting plasmid into HEK293 cells and the stable cell lines expressing the HA at the second extracellular loop of TRPC4 were established as J04. One of them, J04-1, was used for this study.

D. Wild type TRPC4β

Wild type full-length TRPC4β was subcloned into pIRESneo vector. I transfected the resulting plasmid into HEK293 cells and the stable cell lines were designated as A704.

E. Full length TRPC4 and TRPC5 for *in vitro* synthesis

For *in vitro* synthesis of 35S-labeled proteins, the full-length cDNAs of both murine TRPC4 and TRPC5 were subcloned into the expression vector pAGA [Sanford et al., 1991], which contains a T7 promoter that allows transcription initiation by T7 RNA
polymerase. An ATG codon contained within an NcoI site serves as a translation initiation site.

F. The C-terminal constructs for TRPC4α and TRPC5

The C-terminal HindIII/EcoRI fragments of TRPC4α or TRPC5 encoding the amino acid 688-974 (TRPC4α) or 698-975 (TRPC5) were subcloned into pAGA.

G. The C-terminal constructs for PLCβ1 and PLCβ2

cDNAs for bovine PLCβ1 and PLCβ2 were kindly provided by Dr. Peter Gierschik. The C-terminal construct contains the PvuII/BamHI fragment of PLCβ1 (1016-1216) or the PvuII/XbaI fragment of PLCβ2 (927-1181) and was subcloned into pAGA.

H. C-terminal HA-tagged TRPC4 (both α and β) constructs for in vitro synthesis

The HA epitope, YPYDVPDYA, was added to the C terminus of TRPC4α and TRPC4β immediately before the TGA stop codon. The cDNAs were subcloned into pAGA.

I. Construct for TRPC4 N-terminus

A fragment of NcoI/XhoI digestion from TRPC4, coding sequence for amino acid 1-330 aa, was subcloned into pAGA vector for in vitro synthesis of 35S-labeled proteins. The same fragment was also subcloned into pGEX4T-1N vector and used for preparing the glutathione S transferase (GST) fusion protein of TRPC4 N-terminus.

J. Constructs for TRPC3 N-terminus

An N-terminal NcoI/HindIII fragment of TRPC3 coding for amino acid 1-303 was subcloned into pAGA for in vitro synthesis. The same fragment was subcloned into pGEX4T-1N vector and used for preparing the GST fusion protein.
K. HA-NHERF

An N-terminal HA-tagged human NHERF [Murthy et al., 1998] in pcDNA3 vector was kindly provided by Dr. Vijaya Ramesh. This construct was used for transient expression in A172-60 cells.

L. Constructs for NHERF PDZ1 and PDZ2

Constructs for GST fusion proteins of NHERF PDZ1 and NHERF PDZ2 were kindly provided by Dr. Min Li.

2.3.2 DNA constructs prepared by myself

A. TRPC4ΔC

To remove the last four amino acids, TTRL, from TRPC4, a C-terminal fragment encoding murine TRPC4 (A874-V970) followed by a stop codon was amplified by PCR using wild type TRPC4α in pCDNA3 as the template and sense primer 0181 (5'-AGCACTTGAGCGAAATATCG-3') and antisense primer 0470 (5'-ACACATAATCTTCGTGGG -3'). The PCR product was cloned into pCRII vector (Invitrogen). The nucleotide composition of the resulting insert was confirmed by DNA sequencing. A BstEII/EcoRI fragment from wild type TRPC4α in pIRESneo vector was replaced by the BstEII/EcoRI fragment cut from the pCRII vector containing the C-terminal 'TTRL'-deletion. The resulting plasmid was confirmed by sequencing and then transfected into HEK293 cells. Stable cell lines were established as T098. For in vitro synthesis, the BstEII/EcoRI fragment for pCRII vector was used to replace the corresponding fragment for TRPC4 in the pAGA vector.
B. TRPC4ΔC-HAe

In order to add an HA epitope to the second extracellular loop of TRPC4ΔC, a NheI/BspLU11 fragment in TRPC4ΔC-pIRESneo was replaced by the NheI/BspLU11 fragment cut from the TRPC4-HAe construct (Fig. 2). The resulting plasmid was confirmed by sequencing and then transfected into HEK293 cells. Stable cell lines were established as T105.

C. Constructs for TRPC6 N-terminus

cDNA for the N-terminal fragment of murine TRPC6 (M1-A402) was amplified by PCR using mTRPC6 in pCDNA3 as the template and sense primer 0367 (5' - CCATGGTGAGCCAGAGC-3') and antisense primer 0374 (5' - TACGGCAGCAGACCAGAC-3'). The PCR product was cloned into pCRII vector. The nucleotide composition of the resulting insert was confirmed by DNA sequencing. The NcoI (which was added to the region encoding the most N-terminus of TRPC6 and contained the first ATG) and ApaI fragment, in which the 3' overhang of ApaI site was cut by T4 DNA polymerase in the presence of dNTPs, was subcloned into pAGA at NcoI/SmaI sites. To generate a construct for GST fusion protein, an NcoI/XhoI fragment was cut from the above pAGA construct and then subcloned into pGEX4T-1N.

2.4. Semi-quantitative RT-PCR

Total RNA was extracted from different mouse tissues and their concentrations were determined by spectrophotometric measurement. An aliquot of RNA was treated with DNase I in the presence of RNAguard ribonuclease inhibitor. The first strand cDNA was
synthesized by reverse transcription. In brief, RNA was heated at 68°C for 5 min and chilled on ice. The reaction mixture consisted of 5 µg of total RNA, 1 mM dNTP, 1 µl RNAguard, 100 pmol random hexamers, 2.5 mM DTT (1,4-dithiothreitol) and 200 units of Superscript reverse transcriptase (Invitrogen) in a final volume of 20 µl in a buffer containing 50 mM Tris-HCl, 75 mM KCl and 2 mM MgCl₂, pH 8.3 (Invitrogen). The reaction was incubated at room temperature for 10 min to extend the hexamers and then at 37°C for 90 min. The reaction was terminated by heating to 95°C for 10 min followed by chilling on ice. An aliquot of the reverse transcription reaction was used for PCR with primers specific for each TRPC (Table 2). PCR condition was: pre-denaturing at 94°C for 3 min followed by 20 or 25 cycles of DNA denaturing at 94°C for 1 min, primer annealing at 58°C for 45 sec and primer extension at 72°C for 1 min. At the end of the last cycle, the reactions were incubated at 72°C for 5 min and then cooled to 4°C for storage.

To accurately compare the expression level of trpc genes among different tissues, I normalized the amount of the first strand cDNA using β-actin. Limited PCR cycles (25 cycles) were used to amplify β-actin from an aliquot of each RT reaction using primers 0245/0247 (See Table 2). The products were separated by agarose gel electrophoresis and then transferred to nylon membrane (Amersham Pharmacia Biotech.) using standard protocols [Sambrook et al., 1989]. The presence of β-actin products was detected by Southern hybridization using an end-labeled oligonucleotide probe (0246, Table 2), internal to the primers used for PCR. The probe was labeled by [γ-³²P]ATP using T4

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PNK (polynucleotide kinase). The relative amount of β-actin in each tissue sample was determined by phosphoimager. The amount of the cDNA used for a second round of PCR was adjusted accordingly to verify if a similar amount of β-actin product was obtained among all tissue samples. This was repeated three more times to achieve the normalization of all tissue samples. I then amplified *trpc* genes using limited PCR cycles (20 or 25 cycles). Aliquots of products were loaded on 1% agarose gel. Since most of the products were less than 500 bp, the gel was run at constant voltage (95 mV) for 1 hour in 0.5× TBE buffer until the leading edge of the bromophenol blue was in the lower middle part of the gel. The gel was processed for Southern hybridization using oligonucleotide probes internal to the PCR products (Table 2).
Table 2. Oligonucleotides used as primers for PCR and probes for Southern hybridization

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sets (from 5' to 3')</th>
<th>Probe (from 5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>0245 GAGGCCCAAGACCAAGAGGAG 0247 GACAGGATGCAGAAGG</td>
<td>0246 GATGACCCAGATCATGTCTTGAGAC</td>
</tr>
<tr>
<td>trpc1</td>
<td>0236 GATGTCTGGCCAGTCCAGCTCTGATA 0100 TGGTTATATTTGATAGCAAG</td>
<td>0237 ACTTCCAGTTTCACGAGAATTCGG</td>
</tr>
<tr>
<td>trpc2</td>
<td>0186 TGTGTCTCGAATACTCAGACGAG 0187 ACCCAACACACGAGC</td>
<td>0220 GACCGAAGTCTCTTTAGGAG</td>
</tr>
<tr>
<td>trpc3</td>
<td>0221 GCCTTGGGCTCTCCATTCTTCG 0229 GGAAAGCCAGGAACCTGGCT</td>
<td>0228 TTTGAGGCTACACCAAGCT</td>
</tr>
<tr>
<td>trpc4</td>
<td>0154 TCTGCAATATCTCTGGGAAGGATG 0155 AAGCTTTGTTCGAGCAAATTTCCATTC</td>
<td>0166 GTCAGCAGGACAACCAGAGGATGAC</td>
</tr>
<tr>
<td>trpc5</td>
<td>0161 GTAGGAGTTATTATCATATCGGATGAG 0171 CTCTTTATCTACTGTATTACGGCT</td>
<td>0168 CGTTGTATGTCCCAACACATCGGTACCTCCC</td>
</tr>
<tr>
<td>trpc6</td>
<td>0231 GCATTCACACAGTTGAGGA 0232 TTGACCCCTGGATGAGCTCAC</td>
<td>0222 ATCGGCTACGTTCTGTATGGGT</td>
</tr>
</tbody>
</table>

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2.5. Determination of Rat TRPC4 Sequence

A brain from an adult Sprague-Dawley rat was homogenized in 16 ml TRIZOL reagent (Invitrogen-Life tech.) in a polytrone homogenizer. Total brain RNA was prepared following manufacturer’s protocol. First strand cDNA was prepared from 20 μg of total RNA as described above. The primers used for PCR were summarized in Table 3. The PCR condition was the same as described above except that the total reaction cycle was 35 instead of 20-25. PCR products were separated on 1.2 % agarose gels by electrophoresis. Appropriated DNA fragments were extracted with a Gel Extraction kit (Qiagen), subcloned into pCRII, and sequenced using the USB Sequenase 2.0 kit.

2.6. Membrane protein preparation

Cells from five 10 cm dishes with 100% confluence were collected and rinsed with a cold BSS buffer containing 50 μM CaCl₂, 980 μM MgCl₂, 5.4 mM KCl, 126 mM NaCl, 14.5 mM Tris-HCl and 0.01% glucose. After rinsing, cells were homogenized in 10 ml of a homogenization buffer containing 20 mM HEPES, 1 mM EDTA and 27% glucose, by a polytron homogenizer set at the power level of 5-6 for 5 times of 5 seconds each with a 30 second interval. Samples were centrifuged at 300 rpm for 5 min using a Beckman J6B centrifuge to pellet nuclei and cell debris. The supernatant was collected and centrifuged again at 27,000 × g (15,000 rpm using a SS-34 rotor in a Sorvall RC-5B centrifuge) for 30 min at 4°C. The pellet was resuspended in 500 μl homogenization buffer and stored in 50 μl aliquots at -80°C for future use.
Table 3. Oligonucleotides used as primers for isolating rat TRPC4 by RT-PCR.

<table>
<thead>
<tr>
<th>Primer pair #</th>
<th>Primers (5'-3')</th>
<th>Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0364 GCATGGCTCAGTTCTATTAC 0183 AAGTCCGAGGAGTGCCG</td>
<td>351 (49-400)</td>
</tr>
<tr>
<td>2</td>
<td>0364 GCATGGCTCAGTTCTATTAC 0176 AGACAGCTCATCTACCC</td>
<td>736 (49-785)</td>
</tr>
<tr>
<td>3</td>
<td>0258 CCATGGTGCCCAGCCCACGAGGTC 0255 GTAATGCTGGTTGATGAC</td>
<td>658 (552-1143)</td>
</tr>
<tr>
<td>4</td>
<td>0254 CCATGGTGACGTGTTCTTACAAGATTACAGGC 0178 AAAGTCCATTAGATTCACC</td>
<td>329 (1038-1367)</td>
</tr>
<tr>
<td>5</td>
<td>0268 AAGTTATCTGCAAGGAC 0166 TCAGCAGAACCAGAGAGATGAC</td>
<td>749 (1143-1892)</td>
</tr>
<tr>
<td>6</td>
<td>0157 TGCTAGCTTTTGGCAAATGACC 0350TTTTGCTCTCTGATCATG</td>
<td>598 (1630-2228)</td>
</tr>
<tr>
<td>7</td>
<td>0257 CCATGGGCTACCCCAAGCCCAACTCCC 0391 CAGGCCAGAGGACACTTG</td>
<td>652 (2026-2678)</td>
</tr>
<tr>
<td>8</td>
<td>0359 CCATGGGAGGAGAATGTTAAG 0366 CCATAGCTGCTGGAGCAGGC</td>
<td>785 (2246-3031)</td>
</tr>
<tr>
<td>9</td>
<td>0365 GAAAGGAATACGGAACCTTTGAGG 0366 CCATAGCTGCTGGAGCAGGC</td>
<td>256 (2775-3031)</td>
</tr>
<tr>
<td>10</td>
<td>0258 CCATGGTCCACCCACGAGGAGTCC 0253 GAAGACACTGGCGGTGAAG</td>
<td>486 (552-1038)</td>
</tr>
<tr>
<td>11</td>
<td>0217 GCCATATGCACTCTACAA 0176 AAAGTGCCATTAGATTCACC</td>
<td>444 (923-1367)</td>
</tr>
<tr>
<td>12</td>
<td>0268 AAGTTATCTGCAAGGAC 0156 GCTATGATGAATAATTCTTTGGTG</td>
<td>784 (1143-1927)</td>
</tr>
<tr>
<td>13</td>
<td>0157 TGCTAGCTTTTGGCAAATGACC 0376 GGTGAAGGGGTGCGTC</td>
<td>574 (1630 2204)</td>
</tr>
<tr>
<td>14</td>
<td>0377 CCATGGCAGCAGCTTATGAAG 0366 CCATAGCTGCTGGCGGAGGC</td>
<td>960 (2071-3031)</td>
</tr>
</tbody>
</table>
2.7. Preparation of protein extract from mouse brain

The whole mouse brain was washed twice in phosphate-buffered saline (PBS) and then homogenized using a polytron homogenizer in 1 ml RIPA buffer containing 150 mM NaCl, 50 mM Tris-Cl, pH 8.0, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS, 5 mM EDTA supplemented with protease inhibitors [0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamide, 1 µg/ml aprotinin, 10 µM leupeptin, 1 µM soybean protease inhibitor] followed by sonication at full power for 2 minutes using a sonic dismembrator (Fisher Scientific). The homogenate was centrifuged at 5,000 rpm for 15 minutes in cold room. The supernatant was transferred to a new Eppendorf tube. Protein concentration of the supernatant was determined by Bradford method using BCA kit (Pierce).

2.8. Preparation of protein extracts from cultured cells

Cells from one 10 cm dish of 100% confluence were collected and rinsed with PBS. Cells were lysed either in RIPA buffer or TSE buffer containing 150 mM NaCl, 20 mM Tris-HCl, 1 mM EDTA and 1% Triton X-100, by sonication at full power for 2 min. Cell debris were discarded by low speed centrifugation (5,000 rpm, 2 min) at 4°C. The supernatant was collected in a new Eppendorf tube on ice and used freshly.

2.9. Determination of protein concentration by BCA method

All procedures were carried out following the protocol provided by the manufacturer. BSA (bovine serum albumin) was used as standard, which had five concentrations, ranging from 0 to 2,000 µg/ml. 0.1 ml of each standard or unknown sample was added to
appropriately labeled test tubes, while 0.1 ml H₂O was used as a blank. 2.0 ml of the working reagent (to prepare working reagent, 50 parts of BCA reagent A were mixed with 1 part of BCA reagent B) was added to each tube and mixed well. The tubes were incubated at 37°C for 30 minutes. After incubation, they were cooled to room temperature and the absorbance at 562 nm was measured using a Beckman DU-64 spectrophotometer. A standard curve was made according to readings of the BSA standard. The protein concentrations of unknown samples were determined according to the standard curve made on the same day.

2.10. Western blotting

Appropriate amount of protein extract from cells or mouse brains was separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [Laemmli, 1979]. Then the proteins in the gel were transferred to a Hybond nitrocellular membrane (Amersham Pharmacia Biotech.) using a Semi-dry Transfer Blotter (W.E.P. co.) at 120 mA for 2 hrs with three buffers: anode buffer 1 (0.3 M Tris, pH 10.4, 20% methanol), anode buffer 2 (0.025 M Tris, pH 10.4, 20% methanol) and cathode buffer [0.025 M Tris, pH 9.4, 0.04 M ε-aminocaproic acid (Sigma), 20% methanol]. After the transfer, the efficiency of the transfer was checked by staining the membrane with 0.2% Ponceau S for 2 min. The membrane was then blocked for 1 hr with a blocking buffer containing 50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 1% BSA and 5% dry milk. The membrane was then washed three times for 5 min each with TBST buffer containing 50 mM Tris-HCl, 500 mM NaCl and 0.05% Tween-20. Primary antibody diluted in TBST containing 1%
BSA was used to incubate with the membrane overnight at 4°C. The membrane was then washed with the TBST three times for 15 min each. Horse radish peroxidase (HRP)-conjugated secondary antibody diluted in 1% BSA/TBST was incubated with the membrane for 1 hr at room temperature, and the membrane was then washed with TBST three times for 15 min each. The visualization reaction was done using an enhanced chemiluminescence reagent kit (ECL) (Amersham Pharmacia Biotech.) and the signal was detected by exposing to X-ray film (Kodak).

Rabbit anti-TRPC4 N-terminus (T4nAb) and C-terminus (T4cAb) polyclonal antibodies, and mouse anti-actin monoclonal antibody (Chemicon and Sigma), were diluted at 1:2000 (v/v). Rabbit anti-NHERF polyclonal antibody, goat anti-PLCβ (Santa Cruz), goat anti-actin (Santa Cruz), goat anti-ezrin (Santa Cruz), rabbit anti-actin (Sigma), mouse anti-HA monoclonal antibody (12CA5) were diluted at 1:1000 (v/v).

HRP-conjugated anti-mouse and anti-rabbit secondary antibodies were diluted 1000 folds, while HRP-conjugated anti-goat secondary antibody was diluted 10,000 folds.

2.11. Immunohistochemical assays

For perfusion and fixation of mouse brain, mouse was first anesthetized with intraperitoneal injection of 2.5% Avertin. Anesthesia occurred in 5 min. The perfusion pump was primed by running fixative (4% paraformaldehyde/PBS) at a high speed to remove air bubbles from the tubing. The anesthetized mouse was placed on a stage and...
cut open on the chest. A 26-1/2G needle connected to the perfusion pump through the tubing was pushed into lower left part of the heart (the left ventricle of the mouse) and at the same time aorta was cut off and the pump was turned on at an appropriate speed (0.9 ml/min). The transcardial perfusion lasted for 10 min. After the whole heart turned to a greywhite color, the pump was turned off. The mouse was decapitated and the whole brain was placed in 4% paraformadehyde for additional 4-6 hours at room temperature. The brain was cryoprotected in 23% sucrose for 48 hours at 4°C [Baader et al., 1998].

The fixed mouse brain was sectioned on a cryostat at a thickness of 40 μm and sections were removed from the knife and placed immediately into PBS in a 24-well microtiter plate in cold room. Staining was exerted at room temperature. During the staining process, all sections were treated free-floating in wells, and primary antibody binding was revealed using the Vectastain ABC kit according to the instructions of the manufacturer (Vector Laboratories). In brief, to quench endogenous peroxidase activity, sections were incubated in 0.3% H₂O₂ in methanol for 30 min and washed in PBS for 20 min. The floating sections were first blocked for 30 min with 2% goat normal serum in PBST which contained PBS and 0.3% Triton X-100. Primary antibody (T4cAb or peptide absorbed antibody) diluted at 1:100 in blocking solution was incubated with the sections for 2 hrs. Sections were washed three times with PBS for 5 min each and then incubated with diluted biotinylated anti-rabbit secondary antibody solution for 30 min. Sections were washed 3 times with PBS for 5 min each and then incubated with the avidin-biotinylated HRP complex (ABC) for another 30 min. After being washed another 3
times with PBS, sections were incubated in HRP substrate solution (0.3% DAB, 3, 3’-diaminobenzidine) and signals were visualized for 2-7 min until desired images appeared. Then the sections were washed for 5 min with tap water. The sections were placed onto slides and counterstained with cresyl-violet, cleared with histon-clear and mounted using permount. Images were taken using a Zeiss light microscope.

2.12. Treatment of the cells

2.12.1 Thapsigargin treatment

Cells were washed twice with a nominally Ca²⁺-free, Hepes-buffered physiological saline solution (HPSS:116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO₄, 20 mM Na-HEPES, 10 mM glucose, pH 7.4), and then incubated with 1 μM thapsigargin in the same solution for 10 min. The cells were fixed with 4% formaldehyde in Ca²⁺-free HPSS for 30 min. Incubation with primary antibody and subsequent steps were carried out as described in fluorescence staining of permeabilized cells.

2.12.2 Carbachol and ATP treatment

Cells were washed with HPSS with 1.8 mM Ca²⁺ and then treated with 100 μM carbachol and/or 90 μM ATP for 10 minutes in HPSS with or without 1.8 mM Ca²⁺. The treated cells were washed three times with the corresponding Ca²⁺-free or Ca²⁺-containing HPSS and placed on ice. The ice-cold cells were incubated with the anti-HA antibody (1:400) for 1 hr and washed three times with chilled HPSS. Then the cells were fixed with 4%
formaldehyde in HPSS. The staining of HA epitope on the extracellular side of the cells followed the description in fluorescence staining of nonpermeablized cells.

2.13. Fluorescence immunocytochemical staining and confocal microscopy

2.13.1 Fluorescence immunostaining of permeablized cells

All procedures were carried out at room temperature unless otherwise indicated. Cultured HEK293 cells at 20-40% confluence were washed twice with PBS and fixed for 30 min with 4% (v/v) formaldehyde in PBS. The fixed cells were incubated with 100 mM glycine in PBS for 30 min, followed by permeablization with methanol on dry ice for 5 min. Cells were blocked in PBS containing 10% goat serum and 1% BSA for 1 hr and then incubated for another hour in the buffer of the same composition supplemented with primary antibodies. At the end of incubation, cells were washed three times with PBS containing 1% BSA and 0.1% Tween 20 (PBST) and then incubated for 1 hr in the buffer of the same composition supplemented with fluorochrome-conjugated secondary antibodies. After incubation, cells were washed three times with PBST and then mounted with a mounting medium (Sigma). Immunofluorescent signals were examined using a Nikon microscope coupled to a confocal laser scanning unit (Bio-Rad MRC-1000).

2.13.2 Fluorescence immunostaining of nonpermeablized cells

All steps were performed at room temperature unless indicated otherwise. HEK293 cells expressing externally HA-tagged TRPC4 (either control cells or cells treated with carbachol or ATP as described above) were washed twice with PBS. After treatment,
cells were kept on ice and incubated with the anti-HA antibody (1:1000) for 30 min. After the incubation with primary antibody, cells were washed three times with PBS and then fixed with 4% formaldehyde. After being washed another three times, cells were blocked by 1% BSA, 10% goat serum for another 30 min and then stained with Oregon green-conjugated anti-mouse secondary antibody for 15 min. Cells were washed again for three times and mounted with mounting-media and observed under confocal microscope.

2.14. Quantification of cell surface expressed TRPC4

2.14.1 Quantification of confocal images

Immunocytochemical assay and confocal microscopy were used to quantitate the level of the cell surface expressed TRPC4. The stained cell samples were observed under confocal microscope. The green fluorescence images were grabbed using the same level of gain, iris, and background among all the samples. On the image, a line was draw across the cells and the pixel intensity values corresponding to the plasma membrane were recorded as the fluorescence intensities on the cell surface using histogram line program in the confocal computer system. The average pixel intensity values represent the relative amount of the proteins expressed on the plasma membrane of the cells. The comparison among different cell samples was based on the results from the same set of experiments.
2.14.2 Quantification of TRPC4 expression on the plasma membrane by a modified western blotting assay

I utilized a modified immunoblotting assay to quantify the surface HA signals. The purpose of this was to determine the surface expression of TRPC4 in a large population of cells (> 1 x 10^6). Cell lines that express TRPC4 containing an HA epitope at the second extracellular loop were washed twice with PBS and treated with or without carbachol for 10 min. After the treatment, cells were kept on ice and incubated with the anti-HA antibody (1:1000) for 30 min. The stained cells were washed three times and collected in PBS. Cells were broken by sonication in RIPA buffer at full power for 2 min. The anti-HA antibody associated with the cells was brought down by incubation with protein A beads for two hrs. The bound HA antibody was collected by centrifugation at 5,000 rpm for 2 min and washed three times with RIPA buffer at 4°C. After the last wash, 10 µl 2 x Laemmli buffer was added to the pellet and samples were boiled for 5 min. The samples were centrifuged briefly at 14,000 rpm and 8 µl of each sample were subjected to SDS-PAGE. The proteins were then transferred and immunoblotted with an anti-mouse HRP-conjugated secondary antibody directly. The ECL signals were quantified with ImagQuant software (Molecular Dynamics).

2.15. Flow cytometry assay

Same as those in carbachol treatment, cells expressing different mutants of TRPC4 (A727 and J04) were washed with PBS, removed from the dishes by several pipetting, transferred into Eppendorf tubes, and placed on ice. The chilled cells were incubated with
the anti-HA antibody (1:400) for 1 hr. The extracellularly stained cells were fixed with 4% formaldehyde and then washed with ice cold PBS for three times. After washing, cells were incubated with Alexa Fluor 488-conjugated anti-mouse secondary antibody (1:1000) for 30 min at room temperature. The stained cells were washed with PBS for another three times and then subjected to analysis by flow cytometry.

2.16. Immunoprecipitation

2.16.1 Immunoprecipitation for TRPC4, PLC and NHERF

All procedures for immunoprecipitation (IP) were carried out at 4°C. HEK293 cells or mouse brain were lysed in RIPA buffer by sonication as described above. The crude cell lysate (500 μl) was centrifuged at 5,000 rpm in a microcentrifuge for 10 min and the supernatant was transferred to a new Eppendorf tube. Nonspecific binding with protein A Sepharose beads was cleared by incubation with 50 μl of the beads for 1 hr followed by a low speed centrifugation at 5,000 rpm for 2 min. The supernatant was then incubated with appropriate dilutions of the desired antibody for 10-16 hrs. The dilutions for the antibodies were 1:200 for anti-TRPC4 (T4nAb and T4cAb), 1:100 for anti-NHERF and anti-PLCβ1. Immunocomplexes were purified by incubation with 1/10 volume of protein A Sepharose for 2 hrs, centrifuged and washed three times in RIPA buffer. Bound immunocomplexes were eluted by incubating the Sepharose beads in 20 μl 2 x Laemmli buffer at 95°C for 10 min. The samples were centrifuged briefly at 14,000 rpm and 10 μl supernatant was separated by SDS-PAGE. The proteins were then transferred to nitrocellulose membranes and immunoblotted with antibodies as indicated.
2.16.2 Co-immunoprecipitation of TRPC4, ezrin and actin

The procedure for co-immunoprecipitation (Co-IP) of TRPC4 with actin and ezrin was the same as IP described in the previous section except that instead of RIPA buffer, TSE buffer with 1% Triton X-100 was used. After different treatments, cells were lysed as described above and 100 µl of cell lysate were IPed with anti-actin or anti-ezrin antibody at 1:100 at 4°C overnight. The IPed complexes were subjected to western blotting following the previous description (Section 9).

2.16.3 Immunoprecipitation of radio-labeled TRPC4 from different cell lines

Cells (A172-60, A727 and J04) at $2 \times 10^6$ were plated in 6-cm tissue culture dishes at least 16 h before the experiments. Cells were washed once with Hanks' balanced salt solution and then incubated in 1 ml of methionine/cysteine-free Dulbecco's modified Eagle's medium (ICN) containing 5% fetal bovine serum at 37 °C for 1 hr. The medium was replaced by the same medium containing 50 µCi/ml $^{35}$S-express protein labeling mix containing $[^{35}\text{S}]$-Met and $[^{35}\text{S}]$-Cys for 1 hr. At the end of the incubation, cells were rinsed twice with PBS, scraped off from the culture dish in 1 ml of ice-cold PBS containing 1 mM EDTA and 0.1 mM PMSF, and pelleted by centrifugation at 5,000 rpm at 4 °C for 5 min. Cells were then homogenized and the immunoprecipitation was performed as described above. The IPed proteins were eluted in 80 µl of 2 × Laemmli buffer and separated by 8% SDS-PAGE. The gels were stained with Coomassie Blue, destained, and then dried for autoradiography.
2.17. GST Pull-down experiments

2.17.1 Preparation of GST fusion proteins

GST and GST-fused PDZ1, PDZ2 of NHERF and N-termini of TRPC3, 4, 6 proteins were produced in *E. coli* BL-21 cells after transformation with corresponding plasmids. Synthesis of recombinant proteins was induced by the addition of 100 μM isopropyl-1-thio-D-galactopyranoside to log-phase cultures (A600 = ~0.6) at 37°C. After 3-5 hrs of incubation with constant shaking, the bacteria were recovered by centrifugation at 4°C for 15 min and either used immediately or stored at -20°C. GST and GST-fusion proteins were purified, using glutathione Sepharose 4B (Amersham Pharmacia Biotech.), from bacterial lysates prepared by sonication in 20 mM lysozyme and 1% sarcosyl. The glutathione beads together with the bound proteins were washed three times with washing buffer (20 mM Tris-Cl, pH 8.0, 1 mM EDTA, 150 mM NaCl, 0.5% IGEPAL) and then resuspended in a binding buffer containing 20 mM Tris-Cl, pH 8.0, 100 mM KCl, 2 mM MgCl₂ and 0.5% Lubrol.

2.17.2 In vitro synthesis of ³⁵S-labeled proteins

³⁵S-labeled full-length TRPC4, TRPC5, the C-terminal portions of TRPC4, TRPC5, PLCβ1, and PLCβ2, the C-terminal ‘TTRL’-deleted TRPC4, the C-terminal HA epitope-tagged TRPC4, or the N-termini of TRPC3, 4, 6 were made *in vitro* using the TNT (transcription/translation) coupled rabbit reticulocyte system (Promega) in the presence of ³⁵S-methionine following manufacturer’s protocols. Aliquots of the incubation
mixtures were used directly either for analysis by SDS-PAGE to confirm synthesis of proteins of the desired size or for studying the binding between the newly synthesized proteins and GST or GST fusion proteins.

2.17.3 GST pull-down assay

2 μg GST proteins bound to glutathione Sepharose and 10 μl 35S-labeled proteins were added to 250 μl of the binding buffer and incubated on a platform shaker at room temperature for 30 min. The bound proteins were collected by centrifugation at 5,000 rpm for 2 min and washed three times with the binding buffer. After the last wash, 10 μl 2 x Laemmli buffer was added to the pellet and samples were incubated at room temperature for 5 min. The samples were centrifuged briefly at 14,000 rpm and 10 μl of each sample were separated by SDS-PAGE. Gels were stained with Coomassie blue, destained, dried and exposed to X-ray films overnight.

2.18. Manipulation of actin

2.18.1 Separation of F-actin and G-actin by centrifugation

Cell samples were collected by centrifugation at 5,000 rpm for 2 min at 4°C and placed on ice for 5 min. TSE buffer containing 1% Triton X-100 or a modified RIPA buffer (1% Triton X-100, 20 mM HEPES-NaOH [pH 7.2], 100 mM NaCl, 1 mM sodium orthovanadate, 50 mM NaF, 1 mM PMSF, 1 mM aprotinin, 1 mM leupeptin), or a PHEM buffer (60 mM PIPES-KOH, 25 mM HEPES-KOH, 10 mM EGTA, 2 mM MgCl2, pH 7.4) was added to the cell pellet. After the sonication, the cell lysates were carefully
resuspended and subjected directly to centrifugation at 5,000 rpm for 5 or 30 min or 14,000 rpm for 30 min at 4°C in a microcentrifuge, or transferred to ultracentrifuge tubes (Beckman Instruments) and centrifuged at 50,000 rpm (200,000 × g) for 1 hr at 4°C using a Ti-80 ultracentrifuge rotor and Beckman centrifuge. After transering the supernatant to a new tube, the pellet was resuspended in 1/5 volume of the same buffer. Both supernatant (G-actin) and pellet (F-actin) were collected. 10 μl of each fraction brought up to the indicated dilutions (total, pellet, and supernatant) were subjected to 10% SDS-PAGE. The gel was blotted onto nitrocellular membrane and probed with goat anti-actin antibody as described above (Section 9).

2.18.2 F-actin induction by jasplakinolide or phalloidin

Cells were grown under the conditions as described above and treated with or without 1 μM jasplakinolide or 5 μM phalloidin for 2 hrs at 37°C. Cells were scraped off the dishes and lysed in 500 μl TSE buffer as described above. The lysate was spun at 14,000 rpm for 30 min. The supernatant (G-actin) and the pellet dissolved in 50 μl RIPA buffer (F-actin) were detected using western blotting assay. 100 μl of supernatant obtained from centrifuging at 5,000 rpm for 5 min was also used for Co-IP of TRPC4 and actin. Jasplakinolide-treated cells were also used for immunocytochemical staining. Instead of using both primary and secondary antibodies as described in the standard procedure (Section 13), staining of F-actin was done by one-step incubation with Alexa Fluor 488-conjugated phalloidin, which specifically stains F-actin.
CHAPTER 3

RESULTS

3.1. Analysis of TRPC4 expression in native tissues and in heterologously expressed cells

3.1.1 Tissue distributions of TRPC1-6

Ca\(^{2+}\) influx across the plasma membrane plays a vital role in the regulation of diverse cellular processes. The receptor dependent and PLC-mediated formation of inositol 1,4,5-trisphosphate (IP\(_3\)) leads to the depletion of intracellular Ca\(^{2+}\) stores which in turn activates SOCs on the plasma membrane. Mammalian homologues of the *Drosophila* TRP and TRPL ion channels are candidate proteins for store-operated and/or receptor-operated Ca\(^{2+}\) permeable ion channels since TRPC-transfected cells form Ca\(^{2+}\) influx channels activated by store depletion or receptor activation and expression of antisense *trpc* sequences abolished store-operated Ca\(^{2+}\) entry in cultured cells. By 1999, six mammalian *trpc* genes had been cloned and characterized. Later (2000) a new member, *trpc7*, was added to the list.

I tested the distribution patterns for the transcripts of *trpc1-6* in mouse tissues using semi-quantitative RT-PCR followed by Southern blotting (Fig. 3). Equal amount of RNA
Figure 3. Tissue distributions of TRPC1-6 transcripts in mouse. Total RNA from 22 different mouse tissues were extract by TRIZOL reagent according to manufacturer's protocol. First strand cDNA was generated and PCR was performed according to the description in Methods. The indicated genes were amplified for limited PCR cycles (20 or 25). The identities of PCR products were confirmed by Southern blotting using 5'-end radio-labeled synthetic oligonucleotides internal to the primers used for PCR (for primers and probe oligonucleotides, see Table 2). The hybridized bands were visualized by exposing to Kodak X-ray films. Sk, skeletal; Ut, uterus; Adr, adrenal; Pit, pituary; Olf, olfactory; Sm, small.
Figure 3. Tissue distributions of TRPC1-6 transcripts in mouse.
preparations from 22 different mouse tissues, which were normalized by β-actin, were subjected to cycle-limited RT-PCR using specific oligonucleotide primers specific for the six trpcs (Table 2). PCR products were detected using oligonucleotide probes corresponding to each trpcs (Table 2). Transcripts of TRPC1, TRPC2 and TRPC6 were found in almost every tissue while that of TRPC4 and TRPC5 were almost exclusively expressed in neural tissues. The level of TRPC3 transcript is very high in cerebellum. All six trpc genes are highly expressed in neural tissues.

3.1.2 TRPC4 expression pattern in mouse brain

A number of TRPC4 splice variants were identified during the cloning of TRPC4 from different species and tissues [Wissenbach et al., 1998; Merry et al., 2001; Zhang et al., 2001a]. In mouse, rat and human, the most abundant splicing variants, TRPC4α and TRPC4β, differ in an 84 amino acid domain in the cytosolic C terminus of TRPC4α that is absent in TRPC4β [Tang et al., 2000]. I noticed from the RT-PCR results that TRPC4 is almost exclusively expressed in neural tissues, especially in cerebral cortex, cerebellum, olfactory bulbs and midbrain. Therefore, I analyzed TRPC4 expression in mouse brain using immunohistochemistry (Fig. 4). Adult mouse brain sections were incubated with a rabbit polyclonal anti-TRPC4 antibody raised against a synthetic peptide representing the very C-terminal end of bovine TRPC4 as described (T4cAb) [Philipp et al., 2000]. The signals were visualized with 3, 3′-diaminobenzidine (DAB) color reaction. TRPC4 proteins were found in cerebellum, hippocampal CA3 and dentate gyrus region, and olfactory bulbs. These observations are consistent with the
Figure 4. Localization of TRPC4 in mouse cerebellum. Immunohistochemical staining was performed as described in Methods. The brains from neonatal mice were embedded in OCT compound (Tissue-Tek, Miles), and 40 μm frozen sections were transferred to microtiter dishes containing phosphate-buffered saline (PBS). The sections were washed once with PBS and immersed in PBST buffer (PBS plus 0.1% Triton X-100) for 10 min. After blocking with goat serum in PBST, the sections were incubated overnight in 2 μg/ml T4cAb. As a negative control, the anti-TRPC4 antibody was adsorbed with the epitope peptide. (a). The sections were washed in PBST and incubated for 1 hr with biotin-labeled secondary antibodies diluted in PBST. To detect the signal, a universal HRP ABC kit (Vector Lab) was used. The DAB substrate reactions were mounted on slides and visualized by light microscope. Only the cerebellum staining was shown in b (10×). High magnification images (20× in c, 40× in d) show the detailed distribution of TRPC4 protein in cerebellar Purkinje cell soma and dendrites.
Figure 4. Localization of TRPC4 in mouse cerebellum.
result from RT-PCR. Interestingly, TRPC4 immunoreactivity was also abundant in
cerebellar Purkinje cells of the adult mouse. The staining was found in both cell soma and
dendrites (and even axons). However, no immunostaining was detected in the nucleus,
which agrees with the notion that TRPC4 forms plasma membrane channels.

Next I tried to detect the expression of TRPC4 protein in mouse brain using T4cAb. The
expression of TRPC4 in adult mouse brain was not easy to detect by immunoblotting
alone. I therefore performed immunoprecipitation of the mouse brain lysate using T4cAb
and detected the presence of TRPC4 in the immunoprecipitate by immunoblotting with
the same antibody. Two bands, 115 kDa and 105 kDa, presumably representing the α and
β isoforms of TRPC4, were detected (Fig. 5a).

3.1.3 TRPC4 expression in heterologous system

HEK293 cells are capable of serving as an excellent expression system for studying
functional properties of TRPC4 as a receptor-activated Ca\(^{2+}\) channel, inasmuch as they
are known to endogenously express a muscarinic receptor that is coupled to activation of
Gq protein and PLC. Others in the lab expressed mTRPC4α in HEK 293 cells and
established a stable cell line, A172-60. In western blotting, T4cAb recognized a 115 kDa
band from crude membrane prepared from A172-60 but not that from untransfected
HEK293 cells or a cell line (P401-9) stably expressing human TRPC3 (Fig. 5b). I also
established a stable cell line expressing TRPC4β (A704), from which a 105 kDa band
was detected using the same TRPC4 antibody (Fig. 5c).
Figure 5. Western blot detection of TRPC4 in mouse brain and TRPC4 transfected HEK293 cells. 

a. Expression of TRPC4 in mouse brain. A whole brain from an adult mouse was homogenized in 1 ml RIPA buffer as described in the Methods. 200 μl whole brain lysate was immunoprecipitated with T4cAb and the precipitates were separated by 8% SDS-PAGE, transferred, and then probed again with T4cAb. Arrowheads indicate the two bands corresponding to the sizes of TRPC4α and TRPC4β. The identities for the higher molecular weight bands are not known as they appeared in some but not all blots.

b. Expression of TRPC4α was detected in A172-60 but not untransfected HEK293 or TRPC3 transfected (P401-9) cells by western blotting using T4cAb. 2 μg of crude membrane protein prepared as described in Methods was used in each lane.

c. Expression of TRPC4β was also detected in A704 but not HEK293 cells by western blotting using T4cAb.
The distribution of TRPC4α in the A172-60 cells was studied by immunofluorescence confocal microscopy using T4cAb. TRPC4 was evenly localized to the periphery of the A172-60 cells (Fig. 6a), consistent with it being a plasma membrane protein. Under the same condition, untransfected HEK 293 cells or a stable cell line expressing human TRPC3 showed no immunostaining (Fig. 6b&c).

To confirm the plasma membrane localization of TRPC4 proteins, special TRPC4 constructs and the corresponding cell lines were made according to the structure and membrane topology of TRPC4 protein. I established three stable cell lines expressing mutant TRPC4. One is J04, in which a hemagglutinin (HA) epitope was added to the second extracellular loop of the wild type TRPC4. The second is T105, in which a HA epitope was also added at the same site as J04, but the last four amino acids (TTRL) were deleted. The third is A727, which expressed a TRPC4 with an HA epitope added at the most C-terminus right after the last amino acid leucine of TRPC4. I first confirmed the protein expression of different TRPC4 mutants in these cells by immunoprecipitation and western blotting (Fig. 7a, b&c). According to the transmembrane topology determined for TRPC3 [Vannier et al., 1999], both N- and C-termini of TRPCs are located at the cytoplasmic side of the plasma membrane. For J04 and T105, the HA epitope should be exposed to the extracellular side of the plasma membrane whereas for A727, the epitope should be localized at the cytoplasmic side. I tested this idea by immunocytochemical staining of cells with an anti-HA antibody under nonpermeablized conditions. The bound anti-HA antibody was visualized by fluorescence-conjugated secondary antibody.
Figure 6. Distribution of TRPC4 in cells. A172-60 (a), HEK293 (b) or P401-9 (c) cells were fixed, immunolabeled with T4cAb, and stained with Oregon Green-conjugated goat anti-rabbit IgG as described in the Methods. The distribution of TRPC4 in these cells was examined by confocal microscopy. TRPC4 was detected in the periphery of A172-60 cells whereas only background signal was detected in control and P401-9 cells.
Figure 7. Detection of TRPC4 in HEK293 cells transfected with TRPC4 or various TRPC4 mutants.  

a. Expression of TRPC4 in A727 and J04. Immunoprecipitation of [\textsuperscript{35}S] TRPC4 was performed from cell lysates prepared from A727, J04 and A172-60 cells using anti-HA antibody, T4cAb and T4nAb. The names of the cell lines are indicated on the top.  
b. Expression of TRPC4 in T105 cells. 2 \( \mu \)g of crude membrane, prepared as described in Methods was used in each lane. The membrane was blotted with anti-HA antibody, T4nAb and T4cAb. HA signal was only detected in T105 cells. T4cAb could not detect TRPC4 in T105 since part of the epitope, i.e., its C-terminal 'TTRL' was deleted.  
c. Expression of HA-tagged TRPC3 was detected in P401-9 but not in HEK293 cells by western blotting using anti-HA antibody. 2 \( \mu \)g of crude membrane, prepared as described in Methods was used in each lane. J04 cells, in which TRPC4 with HA epitope was expressed, was used as positive control.
Figure 7. Detection of TRPC4 in HEK293 cells transfected with TRPC4 or various TRPC4 mutants.
followed by confocal microscopy. Under these conditions, J04 and T105 cells showed immunostaining, suggesting that both forms of TRPC4 are expressed on the plasma membrane (Fig. 8b&c). A727, which has the HA tag at the C-terminus, did not show immunostaining, indicating that the extracellular staining was specific for the HA epitope exposed to the outside of the cells (Fig. 8a).

The presence of TRPC4 on the plasma membrane was also confirmed by flow cytometry (Fig. 9). Here, the TRPC4 cells were incubated with the anti-HA antibody under nonpermeabilized conditions and then with Oregon green-conjugated secondary antibody. The fluorescence-labeled cells were sorted by fluorescence activated cell sorter. It was clearly shown that J04 cells had in average about 10 times more fluorescence than A727 cells.

3.1.4 Rat TRPC4 sequence

TRPC4 genes have been cloned from mouse, rat, human and bovine. The deduced amino acid sequences for human and bovine TRPC4 share very high homology (97.1% amino acid identity) with an identical amino acid sequence, ‘VTTRL’, at the C-termini [Mckay et al., 2000]. However, the rat sequence, originally called TRP-R [Funayama et al., 1996], was reported to have a difference C-terminal sequence, ‘HKIMI’, and to have 93% overall amino acid sequence identity with mTRPC4α. The nucleotide sequence identity between the mouse and the rat genes is 95%, which is higher than that between mouse and human or mouse and bovine. The high nucleotide sequence identity between the
Figure 8. Plasma membrane localization of TRPC4 in HEK293 cell lines. HEK293 cells expressing different constructs of TRPC4 containing HA epitope at either the C-terminus (A727, a) or the second extracellular loop (J04, b, and T105, c) were washed with PBS twice and incubated with anti-HA antibody (1:400) in PBS for 1 hr at 4°C. After washing, cells were fixed with formaldehyde and then stained with Oregon Green-conjugated anti-mouse secondary antibody (1:1000). The fluorescence signals were visualized by confocal microscopy.
Figure 9. Flow cytometry analysis of Plasma membrane localization of TRPC4 in HEK293 cell lines. A large population of J04 and A727-1 (2 \times 10^6) cells were treated as in Fig.8 and then subjected to flow cytometry as described in the Methods. J04 cells have in average ~10 fold higher fluorescence signal than A727-1 cells.
mouse and rat clones suggests that they most likely belong to the same gene. We therefore carefully inspected the differences between the mouse and rat sequences and found that the lower homology at the amino acid level was due to multiple insertions and deletions in the nucleotide sequence of rat \textit{trp-R}, which resulted in frame shifts relative to \textit{mtrpc4} (Fig. 10). For the sequences that encode the very C-termini of the two proteins, although the nucleotide sequences are 88\% identical, frame shifts not only caused the encoded peptides to be only 29\% identical but also resulted in an earlier termination of rat TRP-R with "HKIMI" instead of "VTTRL" (Fig. 10a). At the same region, bovine TRPC4 and human TRPC4 are both 86\% identical to mTRPC4 at the amino acid level and they all terminate with "VTTRL" [Philipp et al., 1998]. The high degree of homology at the nucleotide level between rat \textit{TRP-R} and m\textit{TRPC4} at this region suggests that rather than alternative exons, equivalent exons are used to encode the very C-terminal ends of the two proteins. However, several mutations have been introduced to the sequence reported for TRP-R, rendering the deduced amino acid sequence for its C-terminus to be divergent from murine, bovine, and human TRPC4.

In order to confirm the presence of TRPC4 orthologue in rat, I isolated total RNA from the brain of an adult Sprague-Dawley rat and performed RT-PCR using primers that would anneal to both \textit{mtrpc4} and rat \textit{trp-R} (Table 3). The nucleotide sequences of the PCR products are 99.4\% identical to that of \textit{trp-R} and 95.5\% identical to \textit{mtrpc4}. Interestingly, the rat \textit{trpc4} sequence obtained from RT-PCR lacks all the insertions and the deletions shown for \textit{trp-R} when aligned with \textit{mtrpc4} (Fig. 10b). These corrections
Figure 10. Comparison of the nucleotide and deduced amino acid sequences of rat TRP-R and murine TRPC4. Three fragments are chosen to highlight the reading frame changes due to deletion or insertion of nucleotides in the published sequence for TRP-R (GenBank Acc#: AB008889). a and b are the sequences from the N-termini and c shows sequences from the C-termini. The nucleotide sequences for mtrpc4, rat trpc4 (rtrpc4) derived from RT-PCR, and TRP-R were aligned manually. '-' indicates nucleotides identical to mtrpc4. '.' indicates gaps introduced to obtain the optimal alignment. The deduced amino acid sequence for mTRPC4 is shown above the alignment and that for TRP-R is shown below. The positions of the amino acid residues are denoted to the right of each sequence. Amino acid sequences for mTRPC4 in a and b are identical to that of bovine TRPC4 whereas that in c is 86% identical to the sequence of the same region in bovine TRPC4. The termination codon for TRP-R and that for mTRPC4 and RT-PCR derived rat TRPC4 are highlighted in boxes.
Figure 10. Comparison of the nucleotide and deduced amino acid sequences of rat TRP-R and murine TRPC4.
greatly improved the amino acid identity between rat TRPC4 and mTRPC4α and restored
the stop codon for rat TRPC4 to the same site as that for murine, bovine, and human
TRPC4 (Fig. 10c). In the region included in Fig. 10, the deduced amino acid sequence of
rat TRPC4 is 95% identical to that of mTRPC4 and both are 86% identical to bovine
TRPC4 in the equivalent region. Out of ten clones that we have sequenced, none
corresponded to the sequence of TRP-R. Therefore, most likely the published sequence
for TRP-R was a result of multiple sequencing mistakes.

3.2. TRPC4 N terminus interacts with the N-terminus of TRPC3 and TRPC6

According to the primary structure of TRPC proteins and their analogy to voltage-gated
channels, TRPC channels are proposed to be tetramers formed of identical or different
subunits. The multimerization of Drosophila TRP and TRPL has been shown to occur
through the interaction of their N-termini [Xu et al., 2000]. Therefore, the N-terminal
region of TRPC subunits may also be involved in multimerization. I tested the binding of
TRPC4 N terminus to the N-terminus of TRPC3 or TRPC6, using in vitro GST-pull
down assay (Fig. 11). GST fusion protein containing TRPC4 N terminus interacted
specifically with in vitro synthesized TRPC3 and TRPC6 N termini but not with a
negative control maltose binding protein (MBP), which were prepared from the
corresponding recombinant cDNAs using a transcription and translation coupled rabbit
reticulocyte lysate system in the presence of 35S-labeled methionine and cysteine.
Therefore, TRPC4 is capable of forming a complex with either TRPC3 or TRPC6 via
interaction at their N-termini.
Figure 11. TRPC4 N-terminus interacts with the N-terminus of TRPC3 and TRPC6. $^{35}$S-labeled TRPC3 and TRPC6 N-termini were incubated with GST fusion of TRPC4 N-terminus bound to glutathione Sepharose and bound proteins were separated by SDS-PAGE (8%) as described in the Methods. Middle panel shows the autoradiogram of input $^{35}$S-labeled proteins and upper panel shows those retained by the GST-fusion protein. Lower panel shows the amount of GST-fusion protein used as revealed by Coomassie blue staining. Sizes (in kD) of molecular weight markers are indicated to the left. $^{35}$S-labeled MBP (maltose binding protein) was used as a negative control.
3.3. TRPC4 interacts with NHERF via the TRL motif located at the most C-terminal end

3.3.1. TRPC4 interacts with the first PDZ domain of NHERF

Random peptide library screening has indicated that the first PDZ domain of NHERF preferentially binds to a C-terminal motif “TRL” [Wang et al., 1998]. Because TRPC4 and TRPC5 terminate with a common sequence “VTTRL” (Fig. 12a), it is possible that they interact with NHERF. I tested this possibility using GST pull-down experiments. Fig. 12b&c show that GST fusion protein containing PDZ1 of NHERF interacted with the in vitro synthesized full-length murine TRPC4 and murine TRPC5. PDZ2 of NHERF interacted with TRPC4 and TRPC5 only very weakly. As described earlier, there are two forms of TRPC4, TRPC4α and TRPC4β. Because the C-terminal ends remain the same, both TRPC4α and TRPC4β interacted with PDZ1 of NHERF (Fig. 17a).

To test the association of TRPC4 with NHERF in vivo, I used the stable HEK293 cell line expressing TRPC4α (A172-60) and TRPC4β (A704). As shown earlier (Fig. 5b), the expression of TRPC4 isoforms can be revealed by western blotting using T4cAb. The expression of endogenous NHERF in these cells was detected from the total cell lysates using an anti-NHERF specific antibody NP1 (Fig. 13a) [Murthy et al., 1998]. The association of TRPC4 with NHERF was examined by co-immunoprecipitation (Co-IP) using NP1 followed by the separation of precipitated proteins by SDS-PAGE and immunoblotting using T4cAb. TRPC4 was detected in the precipitates obtained from...
A172-60 and A704 cells but not the untransfected cells (Fig. 13b&c). As a negative control, no TRPC4 signal was found in the blot made from immunoprecipitation of A172-60 cell lysate using a monoclonal anti-HA antibody, 12CA5 (not shown).

In order to determine whether TRPC4 and NHERF also coassemble in a native tissue, I performed Co-IP experiments using adult mouse brain. Significant amounts of NHERF were detected in mouse brain by western blotting using the NP1 antibody (Fig. 14a). Co-IP experiments showed that the anti-NHERF antibody also precipitated the two isoforms of TRPC4 from the brain lysate (Fig. 14b). An unrelated rabbit polyclonal antibody, anti-HisTag, did not precipitate TRPC4. Thus, TRPC4 and NHERF also coexist in the same complex in mouse brain.

3.3.2 TRPC4 is colocalized with NHERF in the periphery of cells

The association of TRPC4 with NHERF was further tested by immunofluorescence confocal microscopy. To do this, I transiently transfected the A172-60 cells with an NHERF construct that contains an HA epitope at its N-terminus. The HA-tagged protein was detected by a monoclonal anti-HA antibody and visualized using goat anti-mouse IgG antibody conjugated to Oregon Green (Fig. 15a), whereas TRPC4 was detected by T4cAb and visualized using Cy-3-conjugated anti-rabbit IgG (red) (Fig. 15b). Significant amount of fluorescence signal for HA-NHERF was colocalized with that for TRPC4 at or close to the plasma membrane (yellow) (Fig. 15c).
Figure 12. TRPC4 and TRPC5 interact with the first PDZ domain of NHERF. 

a, C-terminal sequences of Drosophila TRP, TRP-like and the seven mammalian TRPC homologues. Shown are the last 15 residues for each TRP. Except for the last five amino acids (underlined) of TRPC4 and TRPC5, the C-termini of TRPC proteins are not conserved. Double underlined sequence in Drosophila TRP had been shown to be critical for the interaction between the Drosophila TRP and INAD [Shieh and Zhu, 1996]. b, interaction of full-length TRPC4 and TRPC5 with PDZ domains of NHERF. 35S-labeled mTRPC4 and mTRPC5 were incubated with GST or GST fusion protein of NHERF PDZ1 or PDZ2 bound to glutathione Sepharose and bound proteins were separated by SDS-PAGE (8%) as described in the Methods. c, NHERF PDZ1 interacts with TRPC4, TRPC5, but not TRPC3. 35S-labeled human TRPC3, mTRPC4, or mTRPC5 was incubated with GST fusion of NHERF PDZ1. Proteins were separated by 8% SDS-PAGE. For both a and b, upper panels show autoradiograms of input 35S-labeled proteins and those retained by GST-fusion proteins. Lower panels show the amount of GST or GST-fusion proteins used as revealed by Commassie blue staining. Sizes (in kD) of molecular weight markers are indicated to the left.
Figure 13. Interaction of TRPC4 with endogenous NHERF in HEK 293 cell lines stably expressing various mTRPC4 constructs. 

a, NHERF was detected in control and A172-60 cells by western blotting using the anti-NHERF antibody, NP1. 10 μl of cell lysate containing 1 mg total protein was added to each lane. 

b, 100 μl cell lysate from different cells as indicated was co-immunoprecipitated (Co-IP) with NP1 as described in the Methods. The precipitates were separated by 8% SDS-PAGE and then probed with T4nAb (which recognizes an N-terminal epitope of TRPC4) to reveal Co-IP of TRPC4 with NHERF from cells expressing the α isoform of TRPC4.

c, a similar experiment as b to test the association of NHERF with TRPC4α (A172-60), TRPC4β (A704) and extracellularly HA-tagged TRPC4α (J04), but not C-terminal 'TTRL'-deleted TRPC4 (T105) and C-terminal HA-tagged TRPC4 (A727).
Figure 14. Coassembly of TRPC4 and NHERF in mouse brain. 

a, expression of NHERF in mouse brain. A whole brain from an adult mouse was homogenized in 1 ml RIPA buffer as described in the Methods. 10 μl (1.4 mg total protein) lysate was separated by 10% SDS-PAGE, transferred and probed with anti-NHERF antibody. 

b, association of TRPC4 with NHERF in brain. 200 μl brain lysate was Co-IPed with antibodies for HisTag, NHERF, or TRPC4. The presence of TRPC4 in the immneprecipitates was examined by western blotting using T4cAb. Anti-HisTag was used as a negative control for Co-IP.
Figure 15. Colocalization of TRPC4 with NHERF in A172-60 cells. In order to perform double labeling, HA-tagged NHERF was transfected into the A172-60 cells. The distribution of NHERF was determined using 12CA5 anti-HA monoclonal antibody and Oregon Green-conjugated goat anti-mouse secondary antibody (a). TRPC4 was detected using T4cAb and Cy-3-conjugated goat anti-rabbit secondary antibody (b). The merged image (c) shows the areas where NHERF and TRPC4 colocalize (in yellow).
3.3.3 The C-terminal end of TRPC4 is critical for the interaction with NHERF

In agreement with the notion that the C-terminal sequences of the target proteins are responsible for the interaction with PDZ domains, the binding with NHERF PDZ1 was also detected when only the C-terminal portion of TRPC4 or TRPC5 was used (Fig. 16). In contrast, human TRPC3 terminates with “MLRCE” and therefore did not interact with NHERF PDZ1 (Fig. 12c). However, when the C-terminus was masked by the addition of an HA epitope, neither TRPC4 isoform interacted with NHERF (Fig. 17a), indicating that the presence and exposure of “TRL” motif at the C-terminal end is essential for the interaction between TRPC4 and NHERF PDZ1. Furthermore, when the last 4 amino acids, ‘TTRL’, were deleted from the C-terminal end of TRPC4, no significant binding with PDZ1 of NHERF was detected (TRPC4ΔC in Fig. 17b).

In order to know whether the C-terminal ‘TRL’ motif is also important for the association of TRPC4 with NHERF in vivo, I performed Co-IP experiments using HEK 293 cell lines expressing the C-terminal ‘TTRL’-deleted TRPC4 (T105) or C-terminal HA tagged TRPC4 (A727). Both constructs failed to bind to NHERF in vitro. After immunoprecipitation with NP-1 antibody, western blotting using anti-TRPC4 N-terminal antibody (T4nAb) did not detect the TRPC4 mutants from the precipitates whereas under the same condition, a 115 kDa band representing TRPC4 was detected in the sample precipitated from A172-60 and J04 cells (Fig. 13c). These results indicate that the ‘TTRL’ motif at the C-terminal end of TRPC4 is crucial for the interaction between TRPC4 and NHERF.

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Figure 16. Interaction of the C-terminal portions of TRPC4 and TRPC5 with NHERF PDZ domains. The $^{35}\text{S}$-labeled proteins containing portions of mTRPC4 and mTRPC5 were indicated by the positions of the amino acids shown in parentheses. Proteins were separated by 12% SDS-PAGE. Upper panels show autoradiograms of input $^{35}\text{S}$-labeled proteins and those retained by GST-fusion proteins. Lower panels show the amount of GST or GST-fusion proteins used as revealed by Commassie blue staining. Sizes (in kD) of molecular weight markers are indicated to the left.
Figure 17. TRPC4 C-terminal ‘TTRL’ motif is important for its interaction with NHERF. 

**a.** NEHRF PDZ1 interacts with TRPC4α and TRPC4β but not C-terminal HA-tagged TRPC4 isoforms. Experiments were performed as described in Methods. 35S-labeled TRPC4 isoforms with or without HA-tag were used. 

**b.** PDZ1 of NHERF interacts with TRPC4 but not a C-terminal ‘TTRL’-deleted mutant. In both **a** and **b**, upper panels show autoradiograms of input 35S-labeled proteins and those retained by GST-fusion proteins. Lower panels show the amount of GST or GST-fusion proteins used as revealed by Comassie blue staining. Sizes (in kD) of molecular weight markers are indicated to the left.
3.4. TRPC4 and PLC are present in the same complex through interaction with NHERF

3.4.1 PLC isoforms interact with NHERF

The C-terminus of bovine PLCβ1 and PLCβ2 contains “TPL” and “SRL”, respectively (Fig. 18a). Based on the results of the random peptide library screening, these sequences may also interact with PDZ1 of NHERF [Wang et al., 1998]. Indeed, GST-pull down experiments showed that 35S-labeled C-terminal fragments of PLCβ1 and PLCβ2 bound to the GST-fusion protein containing PDZ1 but only very weakly to that containing PDZ2 of NHERF (Fig. 18b). Therefore, the in vitro binding studies demonstrated that the first PDZ domain of NHERF is capable of interacting with PLCβ1, PLCβ2, TRPC4, and TRPC5, suggesting that NHERF may serve as a scaffold for the clustering of PLC signaling molecules in mammalian cells.

3.4.2 PLC isoforms form a complex with TRPC4 and NHERF in vivo

HEK 293 cells express sufficient amount of endogenous PLCβ1 that is detectable by a polyclonal anti-PLCβ1 antibody (Fig. 19a). Western blotting also revealed the presence of PLCβ1 in the immunoprecipitates obtained using the anti-NHERF antibody, demonstrating that the endogenous PLCβ1 and NHERF associate with each other in vivo in HEK 293 cells (data not shown). Moreover, in A172-60 cells, PLCβ1 was also Co-IPed with TRPC4 as shown by the detection of the PLC in the immunoprecipitates obtained using T4cAb (Fig. 19b). Interestingly, the amount of PLCβ1 Co-IPed by T4cAb was about the same as that by NP1 (Fig. 19b). Assuming that NP1 and T4cAb had the
same efficiency for the precipitation of the NHERF/TRPC4 complex, this result indicates that most likely, PLCβ1 is also part of the same complex that contains TRPC4 and NHERF. Thus, the three proteins coassemble in HEK cells stably expressing TRPC4.

In order to determine whether these proteins also coassemble in a native tissue, I performed Co-IP experiments using adult mouse brain. Significant amount of PLCβ1 and NHERF was detected in mouse brain by western blotting using the corresponding antibodies (Fig. 14a; Fig. 20a). Co-IP experiments showed that about the same amount of PLCβ1 was precipitated by NP1 and T4cAb (Fig. 20). An unrelated rabbit polyclonal antibody, anti-HisTag, did not precipitate PLCβ1. My Co-IP results showed that except for PLCβ isoforms, PLCγ1 and PLCγ2 also forms complex with NHERF and TRPC4 in cells and in mouse brain (data not shown). Thus, PLC, TRPC4, and NHERF co-exist in the same complex in vivo.

3.5. Intracellular scaffold proteins (actin and ERM proteins) are associated with TRPC4 through NHERF

It has been reported that mTRPC4 and mTRPC5 form nonselective cation channels that respond to activation of G-protein-coupled receptors and receptor tyrosine kinases [Schaefer et al., 2000]. Bovine TRPC4 has been shown to form a relatively Ca$^{2+}$ selective SOC when expressed in HEK 293 cells, Chinese hamster ovary (CHO) cells and rat basophilic leukemia cells [Philipp et al., 1996; Warnat et al., 1999]. In bovine adrenal cortex cells, TRPC4 contributed to the formation of a channel that conducted the Ca$^{2+}$-
bovine PLCβ1 . . ELEGENPGKEFDTPL*  
bovine PLCβ2 . . QDPLIAKADAQESRL*

**Figure 18.** PLCβ1 and PLCβ2 interact with the first PDZ domain of NHERF.  

**a.** C-terminal sequences of bovine PLCβ1 and PLCβ2. Shown are the last 15 residues for each protein. 

**b.** Interaction of PLCβ1 and PLCβ2 with PDZ domains of NHERF.  

35S-labeled C-terminal portions of PLCβ1 and PLCβ2 were incubated with GST or GST fusion of NHERF PDZ1 or PDZ2 bound to glutathione Sepharose and bound proteins were separated by 12% SDS-PAGE. Upper panel shows the autoradiogram of input 35S-labeled proteins and those retained by GST-fusion proteins. Lower panel shows the amount of GST or GST-fusion proteins used as revealed by Comassie blue staining. Size of molecular weights are indicated to the left.

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Figure 19. Coassembly of PLCβ1, TRPC4 and NHERF in A172-60 cells. 

a, detection of PLCβ1 in control (HEK293) and A172-60 cells by western blotting using an anti-PLCβ1 antibody. A 100 kD and a 150 kD form are commonly detected in cell lines and freshly isolated tissues by this antibody and they probably represent alternatively spliced forms of PLCβ1 or proteolytic degradation during sample processing [Dare et al, 1998]. The higher molecular weight bands are common for the HEK cells as they were also seen by others using the same antibody. 10 μl total cell lysate was used in each lane. 

b, PLCβ1 Co-IPed with NHERF and TRPC4. 100 μl of A172-60 cell lysate in RIPA buffer was subjected to immunoprecipitation by NP1 or T4cAb. The precipitates were separated by 8% SDS-PAGE, transferred to nitrocellulose membrane, and probed with the anti-PLCβ1 antibody. About the same amount of PLCβ1 Co-IPed with NHERF and TRPC4.
Figure 20. Coassembly of PLCβ1, TRPC4 and NHERF in mouse brain. 

**a**, expression of PLCβ1 in mouse brain. A whole brain from an adult mouse was homogenized in 1 ml RIPA buffer as described in the Methods. 10 µl (1.4 mg total protein) lysate was separated by 8% SDS-PAGE, transferred and probed with the anti-PLCβ1 antibody as in Fig. 19. 

**b**, PLCβ1 Co-IPed with TRPC4 and NHERF from brain. Brain lysate was immunoprecipitated with antibodies for HisTag, NHERF, or TRPC4. The presence of PLCβ1 in the immunoprecipitates was examined by western blotting using the anti-PLCβ1 antibody.
release activated Ca$^{2+}$ current ($I_{\text{CRAC}}$), the best characterized SOC to date [Philipp et al., 2000]. In Xenopus oocytes injected with rat trpc4 cRNA, Ca$^{2+}$ activated Cl$^-$ current was significantly increased by thapsigargin treatment [Tomita et al., 1998]. Aorta endothelial cells isolated from TRPC4 knockout mice have diminished SOC activity and the aortic ring displayed reduced vasorelaxation in response to acetylcholine [Freichel et al., 2000]. Therefore, most likely mTRPC4 participates in the formation of native SOCs. Recently, it was shown that the function of SOCs as well as that of human TRPC3 stably expressed in HEK 293 cells was dependent on the redistribution of actin cytoskeleton [Patterson et al., 1999; Ma et al., 2000]. This is consistent with the secretion-like coupling model, which suggests that the activation of SOCs is dependent on cytoskeleton-mediated trafficking and exocytotic membrane fusion. It is well known that NHERF interacts with actin-binding ERM proteins [Reczek et al., 1997; 1998]. The finding that TRPC4 interacts with NHERF raises the possibility that TRPC4 is associated with actin cytoskeleton through the NHERF-ERM linkage. Such linkage may play an important role in the activation of TRPC4-based channels.

3.5.1 TRPC4 is associated with ERM$s$ and actin

In the previous section, I showed that TRPC4 formed a complex with PLC$\beta$ and NHERF. The C-terminus of NHERF is known to interact with ERM (ezrin-radixin-moesin) proteins, a group of actin-binding proteins. Therefore, the NHERF-binding proteins should be associated with ERM$s$ and thus with actin, allowing cytoskeleton-mediated trafficking and facilitation of signal transduction.
I tested if TRPC4 protein was associated with ezrin and actin by Co-IP. Initially, I used RIP A buffer to do Co-IP using anti-actin antibody. No TRPC4 was detected in the precipitates by the anti-TRPC4 antibodies. I then used TSE buffer, which contains a milder nonionic detergent, Triton X-100, to do the Co-IP. This time I was able to see TRPC4 in the sample precipitated with either anti-actin or anti-ezrin antibody (anti-ezrin antibody recognizes all members of the ERM family) (Fig. 21). Thus TRPC4 indeed forms a complex with ERMs and actin. The association of TRPC4 with actin or ERMs appears to be weaker than the association between TRPC4 and NHERF as the latter was not disrupted by the use of RIP A buffer, which contains a strong ionic detergent, 1% SDS. This association may be mediated by NHERF since another TRPC protein, TRPC3-HA (P401-9), was not precipitated by either anti-actin or anti-ezrin antibody. I have shown that TRPC3 does not interact with NHERF in both in vitro protein interaction study and in vivo Co-IP study.

3.5.2 TRPC4 is associated with actin and ezrin through interaction with NHERF

I then tested if the association between TRPC4 and actin/ezrin was dependent on TRPC4’s interaction with the adaptor protein, NHERF. Here I utilized A727, a stable cell line that expresses the TRPC4 with an HA epitope added at the most C-terminal end. The addition of HA masked the PDZ binding motif and thus prevented the interaction of TRPC4 with NHERF. Co-IP results showed that in contrast to the wild type TRPC4, the C-terminal HA-tagged TRPC4 could not be precipitated by either the anti-actin or the
anti-ezrin antibody (Fig. 22). This result confirmed that the association between TRPC4 and actin and that between TRPC4 and ezrin are indirect. The C-terminal NHERF binding motif is required for the associations.

To further confirm that the C-terminal residues of TRPC4 are important for the association of TRPC4 with actin and ERM, Co-IP was performed using J04, T105 and T098 cells (Fig. 22). TRPC4 was Co-IPed with anti-actin and anti-ezrin antibodies only from J04 cells, which express the externally HA-tagged TRPC4 containing the intact C-terminal ‘TTRL’ sequence. Mutant TRPC4 proteins with ‘TTRL’-deleted from the C-terminus, such as those expressed in T105 and T098 cells, were not Co-IPed with either actin or ERM. These results suggest that most likely the association of TRPC4 with actin and ERM occurs through the interaction with the PDZ domain-containing protein, NHERF, although it cannot rule out the possibility that C-terminal residues have other mechanisms to facilitate the association with ERM/actin in addition to binding to NHERF.

3.5.3 TRPC4 is associated with F-actin

Actin is an exceedingly complex cellular molecule in terms of its dynamics and interactions with other proteins. This cytoskeletal protein is known to be essential for a wide array of cell functions such as motility, chemotaxis, phagocytosis, macropinocytosis, and cytokinesis, as well as for cell polarity and differentiation [White et al., 1983]. Actin exists in cells in two forms. One is the monomer, which is called G-
Figure 21. The association of TRPC4 and its mutants with actin and ezrin in cell lines. Cells from different cell lines (HEK293 as control, A172-60, P401-9, A727, J04) were coimmunoprecipitated with anti-actin (1:100) and anti-ezrin (1:100) antibodies as described in the Methods. The eluted samples were separated by SDS-PAGE (10%), blotted and detected with T4nAb (1:2000) (a) or the anti-HA (1:1000) antibody (b). The amount of actin in each sample of the cell lysates was detected by western blotting using the goat anti-actin (1:1000) antibody (c). The amount of TRPC4 in each sample was detected by western blotting using T4nAb (d).
Figure 22. The C-terminal TTRL sequence of TRPC4 is necessary for the association between TRPC4 and actin/ERM. a. Cells from different TRPC4 cell lines (A172-60 and J04 expressing TRPC4, T098 and T105 expressing C-terminal TTRL deleted mutants of TRPC4, A727 expressing TRPC4 with its C-terminus masked by an HA-tag) were immunoprecipitated with anti-actin (1:100) and anti-ezrin (1:100) antibodies as described in the Methods. The eluted samples were subjected to SDS-PAGE (10%), blotted and detected with T4nAb (1:2000). b. The amount of TRPC4 in each sample was detected by western blotting using T4nAb (1:1000).
actin (‘G’ for globular); the other one is the polymer called F-actin (‘F’ for filamentous). The two forms convert dynamically back and forth in the cytosol and intracellular ionic strength is critical for the conversion. During the course of this study, we noticed that although a significantly large amount of actin was detected by by western blotting using anti-actin antibodies, after immunoprecipitation, only very small amount of actin was retained. I performed the following experiments to determine why this is the case. The results suggest that most likely anti-actin antibodies preferentially precipitate F-actin.

3.5.3.1 Detection of actin in HEK293 cells using different anti-actin antibodies and different buffer systems

When I performed the Co-IP experiments to test the association between actin and TRPC4, I found that a very low level of actin was precipitated by anti-actin antibodies (a goat antibody from Santa Cruz, S1615, and a mouse antibody from Sigma, A4700) when I used either one of the two common buffers, TSE and RIPA (Fig. 23a). I wondered if the two antibodies possessed low efficiency to precipitate actin. Therefore, I tried two other anti-actin antibodies: a rabbit antibody from Sigma (A2668), and a mouse antibody from Chemicon (MAB1501). Both antibodies recognized the 43 kDa actin band on western blots performed using the total lysate of HEK 293 cells (data not shown). However, after immunoprecipitation by any one of the anti-actin antibodies, very low level of actin was detected (Fig. 23b).
Figure 23. Very low levels of actin were precipitated by anti-actin antibodies. a. A low level of actin protein was precipitated by goat and mouse anti-actin antibodies. HEK293 cells were lysed in RIPA or TSE buffer as described in Methods. 100 μl of lysate was subjected to immunoprecipitation by goat (Santa Cruz) or mouse (Sigma) anti-actin antibodies. The eluted sample was loaded on 12% SDS-PAGE, blotted and detected by goat anti-actin antibody (1:1000). 5 μl cell lysate in RIPA buffer (E) or supernatant (S) and pellet (P) obtained after centrifugation at 14,000 rpm for 5 min in TSE buffer were also loaded as control for total actin. G, goat anti-actin antibody; Ms, mouse anti-actin antibody. b. Low levels of actin protein were precipitated by different anti-actin antibodies. A172-60 cells were lysed in TSE buffer and immunoprecipitation was performed as described in Methods. 100 μl of cell lysate was immunoprecipitated by goat (Santa Cruz as G), mouse (Sigma as Ms), mouse (Chemicon as Mc) or rabbit (Sigma as R) anti-actin antibodies (1:100). The eluted samples were subjected to 10% SDS-PAGE, blotted and detected by the goat anti-actin antibody (1:1000). 5 μl total cell lysate was used as a control to show the amount of total actin (left lane).
I first suspected that the low efficiency of precipitation came from the use of inappropriate protein Sepharose in the immunoprecipitation experiments for antibodies from different sources. Protein A, G* and L* are known to bind to different immunoglobulins from different species with highly variable efficiencies. According to the recommendation of Sigma (Table 4), protein G should be a better choice than protein A to precipitate goat anti-actin antibody, which was used for the Co-IP study. Protein L should have the highest precipitation efficiency for mouse anti-actin antibody. However, I have used protein A Sepharose in all earlier experiments, which could be a reason for the low recovery of actin by the goat or mouse antibody. I thus compared the efficiencies of immunoprecipitation by protein A, G and L conjugated to Sepharose under our buffer conditions. The results showed that protein A Sepharose, if not better than, was at least as good as either protein G or protein L Sepharose in term of the amount of either mouse or goat anti-actin antibodies it could precipitate (Fig. 24a&b).

I then speculated that under my experimental conditions, only F-actin was precipitated by the antibodies. In order to test this possibility, I determined the amount of F-actin in HEK293 cells using a sedimentation protocol for F-actin. I first separated the G-actin from F-actin through a high speed centrifugation (50,000 rpm, 200,000 × g) at 4°C for 30 min (Fig. 25a&b). The supernatant was transferred into another tube. The pellet was dissolved in 1/5 volume of RIPA buffer since it could not be dissolved in the buffer regularly used for Co-IP (TSE buffer) (Fig. 25a). 10 μl of the supernatant and the resuspended pellet were used for western blotting using goat anti-actin antibody.
Table 4. Binding of Immunoglobulins to Protein L, Protein A and Protein G

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<th>Species</th>
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<th>Binding Affinity</th>
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<td></td>
<td>Protein A&lt;sup&gt;++&lt;/sup&gt;</td>
<td>Protein G&lt;sup&gt;+++&lt;/sup&gt;</td>
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<tr>
<td>Human</td>
<td>IgG (Normal)</td>
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Adapted from Sigma catalog, 2002
**Figure 24.** Comparison of protein A, protein G and protein L for the precipitations of goat and mouse antibodies. 

*a.* HEK293 cells were lysed in TSE buffer and incubated with the goat anti-actin antibody as described in Methods. The immunocomplex was precipitated by protein A or protein G beads. The eluted samples were subjected to 12% SDS-PAGE, blotted and detected by the goat anti-actin antibody (1:1000).

*b.* 1 μL mouse monoclonal anti-HA antibody was precipitated by protein A or protein L beads at indicated dilutions. The eluted samples were subjected to 12% SDS-PAGE, blotted to a nitrocellulose membrane, and detected by HRP-conjugated anti-mouse secondary antibody (1:1000).

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<table>
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<th>Buffer:</th>
<th>Extract</th>
<th>IP: Anti-Actin (Goat)</th>
<th>IP: Anti-Actin (Mouse)</th>
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<tr>
<td></td>
<td>P-A</td>
<td>P-G</td>
<td>P-A</td>
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![Image of gel blots](image-url)

**Legend:**
- IgG → Actin →
- Protein A
- Protein L
- Dilution: 1:1, 1:10, 1:50, 1:100, 1:1

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Figure 25. Separation of F-actin from G-actin by high speed centrifugations and the effect of different buffers on maintaining F-actin. 

*a.* separation of F-actin from G-actin by high speed centrifugations. HEK293 cells were lysed in 500 µl TSE buffer and centrifuged at different speed and time as indicated. After removing supernatant, pellets from centrifugation were dissolved in 1/5 volume same buffer. 10 µl of both supernatant and pellet were subject to 10% SDS-PAGE. Actin was detected using goat anti-actin antibody (1:1000). S, supernatant; P, pellet.

*b.* comparison of F-actin centrifuged under different speed. F-actin was collected using the same procedure as in a. Pellet from the indicated speed was dissolved in Lammeli buffer directly. 10 µl of sample was subjected to 10% SDS-PAGE and western blotting.

*c.* comparison of modified RIPA buffer (mRIPA) and TSE effect on maintaining F-actin in the pellet. F-actin preparation and actin detection were the same as in a.
Fig. 25a, lane 6, 8 and 10, and Fig. 25b, lane 2 and 4 show that comparing to the supernatant, very small amount of actin is present in the pellet. I assume that in HEK293 cells, the actin detected from the supernatant is G-actin, while that in pellet is F-actin. This result suggested that very small fraction of actin is present in the filamentous form. However, this analysis must be considered as a crude estimate of how much F-actin is present in the cells. In our sedimentation analyses we made no distinction between free F-actin and the G- or F-actin pools associated with membranes. Even supernatant may conceivably contain actin in various forms that are bound to large protein complexes. These data, therefore only give a quantitative estimate of total pelletable actin. The actual amount of F-actin could be even lower.

To show that the G-actin is not immunoprecipitated by the anti-actin antibodies, I performed immunoprecipitation on the supernatant, which contained a large amount of actin detectable by western blotting. No or very weak signal was detected after immunoprecipitation (Fig. 27b). Because the pellet obtained from high speed centrifugation was very difficult to dissolve in the TSE buffer, which I used for immunoprecipitation, I was unable to determine whether all pelleted actin, presumably F-actin, could be precipitated by the antibodies. However, I tried several means to increase the amount of F-actin. These included treating cells with drugs such as jasplakinolide and phalloidin, a F-actin induction buffer (PHEM buffer), which is composed of 60 mM PIPES-KOH, 25 mM HEPES-KOH, 10 mM EGTA, 2 mM MgCl₂, pH 7.4 and a modified RIPA buffer containing 15 mM HEPES-NaOH pH 7.5, 0.15 mM NaCl, 1% Triton X-100,
1% sodium deoxycholate, 0.1% SDS, 10 mM EDTA, 1 mM DTT, 1 mM sodium orthovanadate, 1 mM PMSF, 1 mM aprotinin, 1 mM leupeptin. Jasplakinolide (JAS) is a cell-permeant cyclic peptide isolated from the marine sponge *Jaspis johnstoni*. It induces polymerization and stabilization of actin filaments in cells [Bubb et al., 1994]. Its actin-binding site and mode of inducing polymerization are highly akin to its structural analog, phalloidin. Phalloidin is isolated from the Death Cap fungus (*Amanita phalloides*). It binds to and stabilizes F-actin. Fluorescent phalloidin derivatives are used to stain F-actin in cells. The PHEM buffer is known to induce actin polymerization in cell lysate in vitro. Except for the modified RIPA buffer, in which low F-actin was maintained after centrifugation (Fig. 25c), all treatments were expected to increase the amount of F-actin. Not surprisingly, more actin was recovered by immunoprecipitation in all cases except when modified RIPA buffer was used (Fig. 26a, b). As shown in Fig. 23b, among all anti-actin antibodies, the Chemicon antibody precipitated more actin from the total cell lysate than the other anti-actin antibodies, suggesting that it may be able to precipitate more forms of F-actin (e.g. α, β or γ actin or a broader extend of polymerization) or some G-actin. Overall, these results indicate that the commercial antibodies preferentially recognize F-actin under my immunoprecipitation conditions although they detect both G-actin and F-actin equally well in western blotting.
Figure 26. Comparison of immunoprecipitation of G-actin and F-actin by the anti-actin antibodies. 

a. separation of F-actin from G-actin by high speed centrifugations and the effect of PHEM buffer on the content of F-actin. HEK293 cells were lysed in 500 μl either PHEM (A) or TSE (B) buffer and centrifuged at different speed and time as indicated. After removing supernatant, pellets from centrifugation were dissolved in 1/5 volume of the same buffer. 10 μl of both supernatant and pellet were subject to 10% SDS-PAGE. Actin was detected using goat anti-actin antibody (1:1000). S, supernatant; P, pellet. For centrifugation at 5,000 rpm, only supernatant was analyzed by the western blotting. 

b. F-actin was induced after treatment by jasplakinolide and phalloidin. A172-60 cells were incubated with 1 μM jasplakinolide (JAS) or 5 μM phalloidin (Ph) for 2 hours and then lysed in TSE buffer. Immunoprecipitation was performed as described in Fig. 21 by goat anti-actin antibody (1:100). Actin in the immunoprecipitates was then detected by western blotting using goat anti-actin antibody.
Figure 27. Comparison of the effect of PHEM buffer and TSE buffer on the immunoprecipitation of anti-actin antibody. 

**a.** The level of TRPC4 Co-IPed by goat anti-actin antibody. A172-60 cells were lysed in TSE buffer or PHEM buffer and the supernatant from centrifugation at 14,000 rpm for 30 min was used for immunoprecipitation as described in Methods. 100 μl supernatant was immunoprecipitated by goat anti-actin antibodies (1:100). The eluted samples were loaded on 10% SDS-PAGE, blotted and detected by western blotting using T4nAb (1:2000).

**b.** The level of actin precipitated by goat anti-actin antibody. The immunoprecipitation and western blotting assay were the same as in a, Actin level was detected by goat anti-actin antibody (1:1000).

**c.** Western Only: Western blotting assay was used to detected the actin levels in the supernatants after centrifugation at 14,000 rpm for 30 min.
3.5.3.2 TRPC4 protein is associated with F-actin

TRPC4 forms channel located on the plasma membrane. After sedimentation, TRPC4 was found in the pellet, where membrane as well as F-actin is presumably present (Fig. 28). I have shown that the Chemicon anti-actin antibody precipitated more actin than antibodies from other sources. However, from the equal amount of the same cell lysate, the Chemicon antibody did not Co-IP more TRPC4 than other anti-actin antibodies (Fig. 29), indicating that TRPC4 is associated with only a subset of actin species, most likely those precipitable by the majority of the anti-actin antibodies. When cell samples were treated with jasplakinolide, phalloidin and the F-actin-inducing PHEM buffer, all of which increased the amount of F-actin in the cells, there was also an increased amount of TRPC4 Co-IPed with the anti-actin antibodies except in PHEM buffer (Fig. 30, only phalloidin effect was shown). These results suggest that TRPC4 is probably only associated with F-actin. This association is most likely dependent on the interaction between TRPC4 and NHERF since in T098 cells, the mutant TRPC4 that does not interact with NHERF was not precipitated by anti-actin antibody even after the F-actin level was increased by the phalloidin treatment (Fig. 31).

I utilized extracellular solutions either containing or not containing Ca^{2+} in my Co-IP experiments for testing the association between actin and TRPC4. I found that the presence of extracellular Ca^{2+} significantly affected the proportion of F-actin in cells. In the absence of Ca^{2+} (nominally Ca^{2+} free buffer), more actin was precipitated by the anti-
Figure 28. TRPC4 is colocated with F-actin after centrifugation. HEK293 cells were lysed in either PHEM (A) or TSE (B) buffer and centrifuged at different speed and time as indicated. Pellets from centrifugation were dissolved in 1/5 volume of the same buffer. Both supernatant and pellet were subject to 10% SDS-PAGE as in Fig 25, a. TRPC4 was detected using rabbit anti-TRPC4 antibody (T4nAb, 1:2000). S, supernatant; P, pellet.
**Figure 29.** About equal amount of TRPC4 was immunoprecipitated by different anti-actin antibodies. A172-60 cells were lysed in TSE buffer and immunoprecipitation was performed as described in Methods. 100 µl cell lysate was immunoprecipitated by goat (Santa Cruz as G), mouse (Sigma as Ms), mouse (Chemicon as Mc) or rabbit (Sigma as R) anti-actin antibodies (1:100). The eluted sample was loaded on 10% SDS-PAGE, blotted and detected by western blotting using T4nAb (1:2000). 5 µl cell lysate was loaded to show the amount of TRPC4 in these cells.
Figure 30. The association between TRPC4 and actin was increased after phalloidin treatment. A172-60 cells were incubated with 5 μM phalloidin (Ph) for 2 hours and then lysed in TSE buffer. Cell lysates were centrifuged as indicated and supernatants were collected. Immunoprecipitation was performed using 100 μl supernatant as described before by goat anti-actin antibody (1:100). TRPC4 in the immunoprecipitate was detected using T4nAb (1:2000). Actin in the same blot was detected by the goat anti-actin antibody (1:1000).
Figure 31. The association between TRPC4 and F-actin was dependent on the C-terminal TTRL motif of TRPC4. J04 and T098 cells were treated with phalloidin and lysed in TSE buffer as described above. F-actin was prepared by centrifugation at 14,000 rpm x 30 min and pellets were resuspended in TSE buffer. Supernatant after cleaning with 2 min centrifugation at 5,000 rpm was also immunoprecipitated by anti-actin, anti-TRPC4 and anti-ezrin antibodies as described before. Both pellet and immunoprecipitated samples were subjected to 10% SDS-PAGE. TRPC4 and actin were detected using corresponding antibodies. C: untreated control; P: phalloidin treated.
actin or anti-ezrin antibody than in the presence of physiological Ca\(^{2+}\) (1.8 mM) (Fig. 32a). This increase could be caused by the conversion of G-actin to F-actin since under my experimental conditions, only F-actin could be precipitated. Phalloidin treatment caused a further increase of immunoprecipitable actin in the absence of extracellular Ca\(^{2+}\). After phalloidin treatment, there is no difference between the amount of actin precipitated in the presence or absence of Ca\(^{2+}\) either with the anti-actin or the anti-TRPC4 antibody (Fig. 32b).

Immunofluorescence staining and confocal laser scanning microscopy were utilized to confirm the association between TRPC4 and F-actin in vivo. Typical prominent F-actin stress fibers were visualized with Alexa Fluor 488 (green)-conjugated phalloidin (Fig. 33b). TRPC4 proteins were stained with T4cAb antibody followed by Cy-3-conjugated secondary antibody (red) (Fig. 33a). F-actin was found to colocalize with TRPC4, which is shown as yellow in the merged images. Moreover, when TRPC4 cells were treated with jasplakinolide to induce actin polymerization, F-actin was found in large clusters near the plasma membrane. Interestingly, significant redistribution of TRPC4 also occurred due to jasplakinolide treatment and the TRPC4 signal always colocalized with that of F-actin (Fig. 33c). The specific association of TRPC4 with F-actin but not G-actin is consistent with the understanding that F-actin is involved in intracellular membrane trafficking, and ERM proteins only interact with F-actin [Bretscher et al., 1997], further supporting the notion that TRPC4 is linked to actin through NHERF and ERMs.
Figure 32. Ca²⁺ removal induced F-actin formation. 

**a.** Removal of extracellular Ca²⁺ increased the level of immunoprecipitated actin. HEK293 cells were incubated in a solution containing nominally-free Ca²⁺ or 1.8 mM Ca²⁺ for 10 min and lysed with TSE buffer. 100 μl cell lysate was immunoprecipitated by goat anti-actin antibody and the eluted sample was subjected to 10% SDS-PAGE. Actin in the immunoprecipitates was detected by goat anti-actin antibody.

**b.** The effect of Ca²⁺ removal and phallolidin treatment on the immunoprecipitable actin. A172-60 cells were treated with or without phallloidin (5 μM) for 2 hours, and then incubated in the nominally Ca²⁺ free or 1.8 mM Ca²⁺ solution for 10 min. Cells were lysed in TSE buffer. 100 μl cell lysate was immunoprecipitated by anti-actin or anti-TRPC4 antibody, and the eluted samples were subjected to 10% SDS-PAGE. Actin was detected by the goat anti-actin antibody.
Figure 33. TRPC4 is associated with F-actin in vivo. A172-60 cells were treated with or without 1 μM jasplakinolide for 2 hours. The cells were incubated with both anti-TRPC4 antibody and Alexa 488-conjugated phalloidin which specifically stains for F-actin (b). The TRPC4 protein was visualized with Cy3-conjugated anti-rabbit secondary antibody (a). The colocalization of TRPC4 and F-actin is shown as yellow in the merged images (c).
3.6. PLC activation and store depletion increased the association of TRPC4 with actin and ERM

3.6.1 Stimulation of TRPC4 channels through the PLC-mediated pathway increased the association of TRPC4 with both actin and ERM

TRPC proteins participate in the PLCβ mediated signal transduction pathway. Since TRPC4 is a part of the huge protein complex that includes PLC, NHERF, ERM and actin and the association of TRPC4 with actin/ERM may be involved in regulating the activity of TRPC4, it is possible that this association is dynamically regulated by the activity of PLC-mediated signaling. I tested this possibility by comparing the amount of TRPC4 Co-IPed with ERM and actin in carbachol-treated cells to that in untreated cells. Carbachol activates PLCβ through endogenous muscarinic receptors and hence causes Ca\(^{2+}\) release and Ca\(^{2+}\) influx in HEK293 cells [Zhu et al., 1998]. Cells were treated with or without 100 μM carbachol in HPSS (Hepes-buffered physiological saline solution) plus 1.8 mM Ca\(^{2+}\) or HPSS with nominally free Ca\(^{2+}\) at room temperature for 1 min. The reactions were stopped by adding 9-fold volume of ice-cold buffer of the same composition without carbachol. Cells were collected, lysed with TSE buffer by sonication and Co-IP was performed using the anti-actin and anti-ezrin antibodies. The result showed that after treatment with 100 μM carbachol, the association between TRPC4 and actin/ERM was increased around 100% (Fig. 34). The carbachol-stimulated increase of association is also dependent on the ability of TRPC4 to interact with NHERF because the association of TRPC4 with actin/ERM was not seen after carbachol treatment in cells that expressed the C-terminal HA-tagged TRPC4 (A727-1, Fig. 34) or the C-terminal ‘TTRL’ deleted...
TRPC4 (T105, not shown). On the other hand, in J04 cells, the TRPC4 containing the HA epitope at the second extracellular loop responded to carbachol with an increase in association with actin/ERM similar to the wild type channel TRPC4α (data not shown).

In addition, the carbachol-induced increase in TRPC4-actin/ERM association is not dependent on the extracellular Ca\(^{2+}\) concentration. Although omitting Ca\(^{2+}\) from the extracellular solution caused a large increase in the association under resting conditions, stimulation with carbachol increased the association further (Fig. 34).

3.6.2 Store-depletion increased the associations of TRPC4 with both actin and ERM

TRPC4 channel may also be activated by store-depletion [Philipp et al., 1996; Warnat et al., 1999]. Thapsigargin (TG) is a plant extract that releases the stored calcium by blocking the intracellular Ca\(^{2+}\)/ATPase [Patterson et al., 1999]. Using the same treatment procedure with 1 μM thapsigargin instead of carbachol and Co-IP experiments as described above, I also showed that about 100% increase was induced by thapsigargin for the association between TRPC4 and actin/ERM in the Ca\(^{2+}\)-containing and nominally Ca\(^{2+}\)-free solutions (Fig. 35). Again, no association between TRPC4 and actin or ERMs was seen after thapsigargin treatment of the C-terminal ‘TTRL’-deleted TRPC4 (T105, Fig. 35)
Figure 34. Effects of Carbachol and Ca²⁺ on the associations of TRPC4 with actin and ezrin. Cells were either untreated or treated with carbachol (100 nM) in the presence or absence of Ca²⁺ as indicated for 1 min at room temperature. The Co-IP was performed and the eluted samples were detected by western blotting using TRPC4 antibody. In a, samples were precipitated with an anti-actin antibody. In b, samples were IPed with an anti-ezrin antibody. In c, western blotting was performed using T4nAb for all the samples to show the relative amount of TRPC4 in each cell lysate.

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Figure 35. Effects of thapsigargin and Ca^{2+} on the associations of TRPC4 with actin and ezrin. Cells were either untreated or treated with thapsigargin (1 μM) in the presence or absence of Ca^{2+} as indicated for 1 min at room temperature. The co-immunoprecipitation was performed and the eluted samples were detected by western blotting using the TRPC4 antibody. In a, samples were precipitated with an anti-actin antibody. In b, samples were precipitated with an anti-ezrin antibody. In c, western blotting was performed with T4nAb for all the samples to show the relative amount of TRPC4 in each cell lysate.
3.6.3 Store-depletion induced clustering of TRPC4

Changes in association of TRPC4 with actin cytoskeleton may be accompanied with changes in the distribution of TRPC4-formed channels on the plasma membrane. Therefore I compared the distribution of TRPC4 in the A172-60 cells before and after thapsigargin treatment. A172-60 cells were incubated for 10 min at room temperature in the absence or presence of 1 μM thapsigargin before they were fixed with 4% formaldehyde for immunocytochemical staining.Ca$^{2+}$ was omitted from the incubation solution to prevent the inactivation of SOC. The untreated cells had an even distribution of TRPC4 on the plasma membrane (Fig. 36a). In contrast, the majority of thapsigargin-treated cells showed punctate fluorescent signals along the periphery of the cells (Fig. 36b), indicating that after stimulation of SOC by thapsigargin, TRPC4 became more aggregated to some discrete sites of the plasma membrane. This suggests that Ca$^{2+}$ influx channels formed by TRPC4 undergo redistribution on the plasma membrane in response to thapsigargin-induced store-depletion.
Figure 36. Clustering of TRPC4 on A172-60 cells after TG treatment. A172-60 cells were incubated in a nominally Ca^{2+}-free buffer in the absence (a) or presence (b) of 1 μM TG for 10 min before being fixed with 4% formaldehyde. Cells were immunolabeled with T4cAb followed by immunostaining with Cy-3-conjugated goat anti-rabbit secondary antibody. Immunofluorescent signals were examined by confocal microscopy. Same laser power, iris and gain were used for both images.
3.7. Stimulation of PLC pathway increased TRPC4 distribution on the plasma membrane

The secretion-like coupling model predicts that the number of SOCs on the cell surface should increase in order for the channels to be activated. To examine whether this is true for TRPC4, I measured the relative amount of TRPC4 protein on the cell surface before and after treatment with 100 μM carbachol. I utilized the TRPC4 construct that contains the HA at the second extracellular loop (J04 cells). The expression of TRPC4 on the plasma membrane was determined by staining non-permeablized cells with an anti-HA monoclonal antibody followed by detection using confocal microscopy as in Fig. 8. Carbachol treatment caused significant increase in the fluorescence signal surrounding the cells (Fig. 37b), suggesting that the amount of TRPC4 on the cell surface is increased as a result of PLC activation. In contrast, the expression of C-terminal ‘TTRL’-deleted TRPC4 (T105) on the plasma membrane was not changed after stimulation with carbachol (Fig. 37), indicating that the interaction with NHERF is important for the increased cell surface expression of TRPC4 protein in response to PLC stimulation.

Many agonists, in addition to carbachol, exert their effect through PLC pathway. I wondered if the redistribution and insertion of new TRPC4 channel onto the plasma membrane is a generous phenomenon in all cases of channel activation through the PLC pathway. I tested the effect of another agonist, ATP, which acts at the P2Y receptor expressed in the HEK293 cells. Electrophysiological studies have shown that TRPC3 was activated by ATP (90 μM) [Hurst et al., 1998]. I treated cells with ATP using the same
protocol as that in the carbachol treatment. Surprisingly, I found that the level of surface expressed TRPC4 was decreased instead of being increased after the ATP treatment (Fig. 38). Considering that the P2Y receptor desensitizes much faster than the muscarinic receptor [Mundell and Benovic, 2000], it is possible that the treating period (10 min) was too long to keep the increased TRPC4 on the plasma membrane due to the fast internalization of the TRPC4 channels.

Because the extracellular Ca\(^{2+}\) concentration greatly affects the association between TRPC4 and actin/ERM, I also tested if extracellular Ca\(^{2+}\) is important for the carbachol-stimulated insertion of new TRPC4 protein to the plasma membrane. The results showed that extracellular Ca\(^{2+}\) concentration has no effect on the redistribution of TRPC4 for both carbachol- and ATP-treated cells (Fig. 39, only carbachol effect was shown).

In order to quantify the differences, I measured the pixel intensities of the fluorescence signal at the cell peripheral for multiple images obtained from carbachol-treated and untreated samples using the same laser power, iris and gain. This semi-quantitative measurement indicated that the amount of TRPC4 on the cell surface doubled upon stimulation by carbachol (Fig. 40). More quantitative measurements were performed on >10\(^6\) cells using a modified western blot assay. Carbachol treated or untreated cells were chilled to 4\(^\circ\)C and incubated with the anti-HA antibody for 1 hrs. After extensive wash, the cells were homogenized and the amount of antibody retained by the cells was determined by western blotting using the anti-mouse secondary antibody only. Under
these conditions, the HA antibody was detected in samples prepared from the J04 and T105 but not A727 cells (Fig. 41a). Carbachol caused a 1.3-fold increase in the amount of antibody retained by J04 cells but no increase in the T105 cells (Fig. 41c). This confirms the results obtained using confocal microscopy.
Figure 37. Carbachol treatment in a nominally Ca\(^{2+}\) free solution increased the fluorescence signal on the external surface of J04-1 cells but not T105 cells. J04-1 (a) and T105 (b) cells were untreated or treated with carbachol in the Ca\(^{2+}\) free buffer and then immunostained extracellularly with anti-HA antibody (1:1000) followed by Oregon Green-conjugated anti-mouse secondary antibody (1:1000). Immunofluorescent signals were examined by confocal microscopy.
Figure 38. ATP treatment decreased the fluorescence signal on the external surface of J04 cells. J04-1 cells were untreated (a) or treated with ATP (90 μM) (b) in the Ca\textsuperscript{2+} free buffer and then immunostained extracellularly with the anti-HA antibody (1:1000) followed by Oregon Green-conjugated anti-mouse secondary antibody (1:1000). Immunofluorescent signals were examined by confocal microscopy.
Figure 39. Extracellular Ca\textsuperscript{2+} did not affect the expression of TRPC4 on the plasma membrane. J04 cells were treated with CCh (100 μM) in a nominally Ca\textsuperscript{2+} free (a) or a 1.8 mM Ca\textsuperscript{2+}-containing solution (b) and then immunostained extracellularly with the anti-HA antibody (1:1000) followed by Oregon Green-conjugated anti-mouse secondary antibody (1:1000). Immunofluorescent signals were examined by confocal microscopy.
Figure 40. Comparison of the pixel intensities of fluorescence signals of externally anti-HA stained J04 and T105 cells with and without CCh treatment. The confocal images grabbed using the same gain, iris and background were subjected to line intensity analysis using histogram in confocal microscope. The values of pixel intensity on the plasma membrane were recorded and averaged for each cell. The results shown are means ±SEM of 100 cells pooled from four separate experiments.
Figure 41. Carbachol stimulation increased cell surface expression of TRPC4 shown by immunoblotting assay. Cells on 100 mm dishes were treated as indicated and stained extracellularly with the anti-HA antibody (1:400) for 1 hr on ice. Cells were washed with ice-cold PBS and lysed in RIPA buffer. Anti-HA antibodies were precipitated with protein A beads and detected by western blotting using anti-mouse secondary antibody. In a, the HA antibody was detected by immunoblotting assay. In b, the level of TRPC4 protein in different samples were shown. In c, relative densities of each band in a were quantified by the ImageQuaNT software. Data are average ±SEM of four experiments. *p<0.01 different from control by Student t test.
CHAPTER 4

DISCUSSION

4.1. Differential tissue expression of mammalian TRPC homologues

Among the multiple pathways that modulate the changes in intracellular Ca\(^{2+}\) levels, CCE attracts a great deal of attention in the recent years. The molecular compositions of SOCs as well as their activation mechanisms remain to be clarified. Owing to the molecular cloning and functional expression of mammalian TRPC homologues, evidence has accumulated to support the role of TRPC proteins in the formation of SOCs in various cell types [Li et al., 1999; Liu et al., 2000; Philipp et al., 2000]. In this study, I showed by semi-quantitative RT-PCR that mammalian *trpc1*-6 genes were ubiquitously expressed although at various levels across different tissues. Nearly all of them are most abundantly expressed in the brain but are barely detectable in liver and many peripheral tissues. These observations are difficult to reconcile with the assumption that CCE is predominantly observed in non-excitable cells and that hepatocytes represent some of the best models for CCE. The contradictions may come from the reason that mRNA levels do not always equate with protein levels owing to different modes of regulation, and that different cells may require different amount of protein products encoded by these genes.
Therefore, the low level of expression of \textit{trpc} genes in non-excitable cells may simply reflect the cell type-specific requirements for these proteins.

My results also showed that multiple \textit{trpc} genes are coexpressed in the same tissues. Some are abundantly expressed at comparably high levels, suggesting that coexpression may be functionally relevant. Whether the coexpressed proteins encoded by these genes participate in the formation of same channel or are subunits of different channels found in the same tissues remains a question.

4.2. TRPC4 forms Ca\textsuperscript{2+} influx channels on the plasma membrane, probably through multimerization with other members of the TRPC family

Even though TRPC4 has been reported to form non-selective cation channels, it remains controversial whether TRPC4 is involved in CCE. While two groups have demonstrated thapsigargin-stimulated TRPC4 activity [Philipp et al., 1996; Tomita et al., 1998], another group showed that the channel only responded to receptor agonist but not store-depletion by thapsigargin [Schaefer et al., 2000]. Using TRPC4-specific antibodies and specially designed externally HA-tagged TRPC4 construct, I directly showed that mTRPC4 was expressed and localized on the plasma membrane of the transfected HEK 293 cells, which is consistent with its role as a plasma membrane channel. TRPC4 protein is predominant in neuronal tissues, such as cerebellum, hippocampus and olfactory bulbs, which indicate that TRPC4 channel is probably involved in neuronal responses. Equal amount of TRPC4\textalpha and TRPC4\textbeta were expressed in mouse brain, which indicate that
both α and β splicing variants may be involved in forming channels in brain. The 84 amino acid C-terminal domain that distinguishes TRPC4α from TRPCβ contains two CaM binding sites [Tang et al., 2001; Trost et al., 2001]. Since CaM is known to regulate channel activity in response to rises in intracellular Ca\(^{2+}\) concentration, it is possible that the two isoforms differentially regulate Ca\(^{2+}\) homeostasis in neuronal cells. Taken together with the fact that expression of TRPC4 protein in fetal mice is higher than that in adult mice (Unpublished data), it is perceivable that the expression of TRPC4 is temporally and spatially regulated in mouse, which is similar to that of TRPC3 [Li et al., 1999].

Others have shown functionally that coassembling of TRPC1 and TRPC4 formed a novel cation channel that was different from that formed by either TRPC1 or TRPC4 alone [Strubing et al., 2001]. Since TRPC4 N-terminus has the ability to interact with the N-termini of both TRPC3 and TRPC6 as shown by my results, it is possible that TRPC4 forms CCE channel through multimerization with other members of the TRPC family through direct interaction at their N-termini.

4.3. Secretion-like coupling process is involved in the activation and regulation of TRPC4 channels

There are three major hypotheses proposed for the mechanisms of activation of SOCs. One is diffusible Ca\(^{2+}\) influx factor model, in which following the discharge of the stores, a diffusible signal is released from the ER and activates plasma membrane SOCs. The
second is secretion-like coupling model, in which depletion of stores causes fusion of vesicles containing the channels with the plasma membrane. The third, at present, the most favored model is conformational coupling, in which a mechanism of communication between the ER and the plasma membrane is established via a direct protein (Ca\(^{2+}\)-releasing channel proteins on ER)-protein (Ca\(^{2+}\)-influx channel proteins on the plasma membrane) interaction. Although, large efforts are being made to characterize the TRPC channels in various expression systems and to compare their function with that of endogenous SOCs, heterologously expressed TRPC channels have also been used to help to elucidate the activation mechanism of SOCs.

A large body of evidence supporting the conformational coupling hypothesis has emerged. Of particular significance are HEK 293 cells stably expressing human TRPC3 channel. In excised inside-out patches, the activity of TRPC3 was dependent on the presence of IP\(_3\), just like the native miniature IP\(_3\)-activated plasma membrane Ca\(^{2+}\) channels found in macrophages and A431 epithelial cells [Kiselyov et al., 1998; 1999]. Using TRPC3, which has more than 10 times larger single channel conductance than the endogenous SOCs, Kiselyov and colleagues demonstrated that the requirement for IP\(_3\) was dependent on the presence of IP\(_3\) receptors, the ER Ca\(^{2+}\)-release channels which were nevertheless remained to be associated with the excised patches [Kiselyov et al., 1998]. Physical interaction between IP\(_3\) receptors and TRPC3 was later demonstrated by Co-IP [Boulay et al., 1999; Kiselyov et al., 1999] and the interaction domains were identified by GST-pull down experiments [Boulay et al., 1999]. Thus, the functional and biochemical
data obtained by using TRPC3 as a surrogate provide experimental evidence for the conformational coupling hypothesis [Berridge, 1995; Irvine, 1990]. More recently, the importance of functional IP$_3$ receptors on the activation of native SOC and heterologously expressed TRPC3 was also demonstrated in fluorescence measurements of another stable TRPC3 cell line by the use of cell-permeable IP$_3$ receptor inhibitors [Ma et al., 2000]. In the HEK 293 cells, little Sr$^{2+}$ influx occurred through an endogenous SOC pathway activated by thapsigargin whereas TRPC3 channel conducted significant amount of Sr$^{2+}$ influx in response to receptor stimulation [Zhu et al., 1998; Ma et al., 2000]. The authors utilized the distinctive permeability displayed by TRPC3 to show that both the endogenous CCE pathway and TRPC3 activity were blocked when IP$_3$ receptors were inhibited [Ma et al., 2000].

However, evidence supporting the secretion-like coupling hypothesis has also been obtained with TRPC3-expressing HEK293 cells. One group demonstrated that actin redistribution played a critical role in activating CCE as well as TRPC3, substantiating an earlier work done on cultured smooth muscle cells [Patterson et al., 1999]. Evidence for the secretion-like coupling hypothesis also came from electrophysiological studies showing that the ability of a membrane patch to expand was crucial for the activation of SOCs within the patch [Yao et al., 1999]. Inhibition of vesicular trafficking with Rho and a dominant-negative form of SNAP-25 inhibited, whereas an inhibitor of Rho, C. botulinum C3 transferase, increased the activity of SOCs [Yao et al., 1999]. Since the targets of calyculin A and Rho are thought to be actin-binding proteins of the
ezrin/radixin/moesin (ERM) family, these data also indicate that ERM proteins are involved in activating SOCs. Together, these works provided support for the trafficking/translocation mechanism of CCE activation.

Since neither IP$_3$ receptors nor SOCs are freely diffusible in the cytosol, the conformational coupling mechanism and the trafficking/translocation (or secretion-like coupling) hypothesis appear to converge at the point that communication between the store-depletion signal coming from ER and the channels on the plasma membrane requires both protein-protein interaction and movement of the ER components closer to the plasma membrane. One possibility is that plasma membrane channels stay unchanged while the ER membrane comes closer. Another possibility is that both the plasma membrane and ER components move coordinately to discrete sites to allow the gating through conformational coupling. It has been shown in vascular endothelial cells, ATP stimulated Ca$^{2+}$ waves initiated from caveolae [Issiki et al., 1998], specialized microdomains of plasma membrane that are highly enriched of signaling molecules [Okamoto et al., 1998]. Consistent with this idea, TRPC1 was localized to the plasma membrane microdomains enriched of caveolin [Lockwich et al., 2000]. Thus, TRPC homologues have made a significant impact on resolving the activation mechanism of SOC.
4.3.1 TRPC4 forms a large signaling complex with PLC, NHERF, ERM and actin

Studies of Drosophila phototransduction system has revealed that molecules involved in a PLC mediated pathway are clustered through binding to a common multi-PDZ-domain containing proteins, INAD [Chevisich et al., 1997; Tsunoda et al., 1997; Van Huizen et al., 1998]. This association appears to be crucial for the proper localization of the cascading enzymes as well as for the precision of the spatial and temporal control of signal transduction, for instance, the recruitment of G protein upon stimulation [Bahner et al., 2000] and the inactivation of TRP by PKC-mediated phosphorylation [Huber et al., 1998; Liu et al., 2000]. PDZ domain-containing proteins also serve important functions in mammalian cells. At postsynaptic membranes, ion channels and other signaling molecules are clustered via interaction with PSD-95 and/or other multi-PDZ domain containing proteins [Faning and Anderson, 1999]. NHERF was first isolated as a cofactor required for protein kinase A-mediated inhibition of renal brush border membrane NHE3 [Weinman et al., 1995; Yun et al., 1997] and later determined to be the same as the phosphoprotein that binds members of ERM proteins [Reczek et al., 1997; Murphy et al., 1998]. NHERF-2 (also called E3KARP, SIP-1 or TKA-1) is closely related to NHERF and has the same domain structure and similar function [Hall et al., 1997; Yun et al., 1997; 1998]. Both proteins are ubiquitously expressed with higher levels in non-excitabie tissues than excitabie ones [Reczek et al., 1997; Yun et al., 1997]. The functional diversity of NHERF has recently attracted a lot of attention because of the findings that its first PDZ-domain interacts with a number of G-protein-coupled receptors and ion transporting proteins, such as CFTR, sodium-bicarbonate transporters, and H⁺-ATPase.
Analysis of the sequence requirement for NHERF PDZ1 binding also suggested PLCβ1 as a potential ligand [Hall et al., 1998]. Indeed, I show here that the C-termini of both PLCβ1 and PLCβ2 bind NHERF PDZ1. The finding that TRPC4 and TRPC5 also bind to the same domain suggests that NHERF is a potential scaffold for clustering PLCβs and TRPCs. My Co-IP studies further demonstrated the association of PLCβ1, TRPC4, and NHERF in the same complex in HEK 293 cells stably expressing mTRPC4 and in mouse brain. Because the ligand specificity of PDZ domain interaction is determined, in most cases, by the last 3 to 4 C-terminal amino acids of the target proteins, it is predictable that only a subset of mammalian TRPCs can interact with NHERF.

PLCβ3 has also been reported to specifically interact with NHERF-2 through its C-terminal PDZ-binding motif in neuronal tissues [Suh et al., 2001]. NHERF-2 upregulated the activity of PLCβ3 stimulated by carbachol. The specificity of the interaction between PLCβ isoforms and PDZ-containing proteins (PLCβ1 and PLCβ2 with NHERF, PLCβ3 with NHERF-2) and the differential expression of PDZ-containing proteins may be responsible for the diversity of the PLC signaling induced by G-protein-coupled receptors. In addition, yeast two-hybrid screening found that NHERF-2 was a specific PDZ partner for plasma membrane Ca^{2+} -ATPases (PMCA) 2b [DeMarco et al., 2002]. This interaction may allow the functional assembly of PMCA in a multiprotein complex,
facilitating integrated cross-talk between local Ca\textsuperscript{2+} influx and efflux. Therefore, NHERF and its close relative, NHERF-2, play critical roles in spatial and temporal regulation of Ca\textsuperscript{2+} signaling such as Ca\textsuperscript{2+} influx, sensing, buffering and extrusion through the assembly of multiprotein complexes.

NHERF is also known as ERM-binding phosphoprotein of 50kDa (EBP-50) since the carboxyl terminal region of NHERF binds to amino-terminal domains of ERM proteins [Reczek et al., 1997; 1998]. My Co-IP results confirmed that TRPC4 is associated with actin and ERM proteins through NHERF. Disruption of the TRPC4’s binding ability to NHERF resulted in a marked loss of its associations with F-actin, indicating that NHERF may be the only link of the TRPC4 channel to the actin cytoskeleton. My results also show that NHERF binding domain is required for agonist-stimulated increase of TRPC4 on the cell surface. Thus, NHERF may modulate TRPC4 activity by mediating its association with actin network. Ezrin or other members of the ERM family, which bind both NHERF and F-actin, are ideally suited to mediate the interaction. Formation of a signaling complex should allow TRPC4-formed channel to response to the activators with high efficiency. Thus, NHERF is considered one of the functional analogs of INAD. Several other PDZ-domain-containing proteins may exist for the clustering of other TRPCs.

The domain structure of NHERF is different from that of INAD. The latter has five PDZ-domains to which PLC and TRP bind differently. NHERF has only two PDZ domains
and PLCβs and TRPC4/5 all bind to PDZ1. It is thus unlikely that a single NHERF molecule is occupied simultaneously by PLCβs and TRPCs. Rather, it is possible that the molecules are clustered via NHERF's association with ERMs, which oligomerize among themselves. In such a case, stoichiometry for PLCβ, NHERF and TRPC4 may vary greatly. In addition, INAD is a photoreceptor specific protein that is predominantly involved in scaffolding and modulating the photosensing signaling pathway whereas NHERF appears to participate in multiple signaling pathways, as shown by its interaction with multiple types of receptors and ion transporters. Therefore, questions remain as to how NHERF selects among its many suitors and what the physiological significance of each association is. The answer to the first question may lie partly on the tissue specific distribution of each target protein. However, in the case of PLCβ and TRPC4, some kind of sorting mechanism would have to be incorporated so that the binding with one partner does not overwhelm the association with the others. For the second question, answers common to all target proteins would include preferential distribution to the apical membrane of polarized cells [Mohler et al., 1999; Moyer et al., 1999], facilitation of phosphorylation by protein kinase A [Weinman et al., 2000], which associates with ERM [Dransfield et al., 1997], and by non-receptor tyrosine kinase through association with YAP65 by NHERF [Mohler et al., 1999]. However, for each protein that associates with the actin network through interaction with NHERF, specific physiological function remains to be elucidated.
4.3.2 Dynamic associations between TRPC4 and intracellular scaffold proteins and actin cytoskeleton may be involved in activation of TRPC4 channel

By treating cells with thapsigargin or carbachol and performing Co-IP experiments, I showed that the associations of TRPC4 with actin and with ERM proteins were enhanced in response to PLC activation and Ca\(^{2+}\) store depletion. This finding is consistent with the previous report that actin/ERM mediated trafficking is involved in the activation of SOCs [Patterson et al., 1999].

Secretion-like exocytosis is one possible way F-actin and scaffolding proteins exert their effects on channel regulation. The association with actin/ERM may be required for transporting TRPC4 from intracellular vesicles to the cell surface. My data show that after PLC\(\beta\) stimulation, there is no change of the total amount of TRPC4 protein in the cell lysates. Therefore, the increased surface expression of TRPC4 is not due to an increased expression of the total protein content. It is more likely that at the resting state, a large proportion of TRPC4 stays inside the cell, close to the plasma membrane and associated with secretory-like vesicles. After activation, TRPC4-containing vesicles are inserted to the plasma membrane with the help of F-actin. Accordingly, channel activation should be accompanied with an increase in the number of channels on the plasma membrane. Results of immunofluorescence and immunoblotting assay using external labeling of an HA-tagged TRPC4 construct showed significant increase of TRPC4 on the plasma membrane after PLC\(\beta\) activation, all of which are in agreement with this prediction.
It remains undetermined how cytoskeleton modulates the activity of channels. Actin cytoskeleton is very critical for the trafficking of the membrane protein since actin forms the “traffic track”, the microfilament that facilitates the movement of TRPC4 to the membrane after activation. Using immunofluorescence staining and confocal microscopy I demonstrated that TRPC4 is colocalized with F-actin inside the cells. TRPC4 is also redistributed and partially colocalized with F-actin after jasplakinolide treatment, which increased the formation of F-actin. I also found that after carbachol stimulation, the F-actin level was increased. Likewise, depolymerization of F-actin may be involved in the inactivation of TRPC4. Thus the function of TRPC4 may be tightly regulated by actin through dynamic cycles of actin polymerization and depolymerization. On one hand, the actin cycle may be important for the insertion of TRPC4 on the plasma membrane or removal of TRPC4 from it. On the other hand, the reorganization of cytoskeleton could activate Ca\(^{2+}\) entry by allowing the coupling between the ER and the plasma membrane. The formation of F-actin would facilitate the ER-plasma membrane coupling and the depolymerization of F-actin could disrupt the coupling.

TRPC4 activity is also regulated by calcium. Both facilitary and inhibitory effects of Ca\(^{2+}\) have been shown for TRPCs [Lintschinger et al., 2000; Zhang et al., 2001]. It is not clear how Ca\(^{2+}\) facilitates the activation of TRPC channels. However, its inhibitory effect may be carried out by CaM through direct binding to TRPC C-terminus in response to the influx of Ca\(^{2+}\) through the channel itself. In the presence of Ca\(^{2+}\), the effect of carbachol
on the associations between TRPC4 and actin/ezrin is not as obvious as in its absence. This is probably due to the profound inactivation of TRPC4 channel caused by Ca$^{2+}$. The physiological function of TRPC4 channel is to increase intracellular calcium concentration as quickly as possible. Once the intracellular calcium concentration is high enough, the channels are closed. Therefore, a high extracellular Ca$^{2+}$ concentration may trigger the close of the TRPC channel very fast. This will be accompanied with the dissociation of TRPC4 from the actin network. TRPC4 protein will then undergo endocytosis to reduce its number on the cell surface.

4.3.3 Secretion-like coupling is involved in the activation of TRPC4 channels

I have shown for the first time physical evidence that a SOC is linked to the actin cytoskeleton through interaction with NHERF, which in turn binds to members of the ERM protein family and through which it is associated with F-actin. NHERF is the bridge to link membrane channel protein TRPC4 and intracellular cytoskeleton. The amount of TRPC4 on the plasma membrane was increased after the stimulation of PLC signaling pathway by an agonist (carbachol), which agrees with the prediction of the secretion-like coupling hypothesis. My result showing redistribution of TRPC4 on the plasma membrane upon thapsigargin stimulation also present a direct demonstration that a plasma membrane Ca$^{2+}$ influx channel moves in response to store-depletion.

The underlying mechanism of TRPC4 redistribution may be explained by its association with NHERF, which is linked to actin cytoskeleton via binding to ERM proteins. It

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remains to be determined whether there is a direct correlation between the increased association with actin and the enhanced expression of TRPC4 on the cell surface after carbachol stimulation or the redistribution of TRPC4 after thapsigargin treatment. Noticeably, the increased TRPC4 expression on the plasma membrane by carbachol stimulation was abolished when the C-terminal NHERF-binding site was deleted or masked with the HA epitope, indicating that the interaction with NHERF plays an important role. Previous data have shown that blocking dephosphorylation of ERM proteins led to the accumulation of F-actin to the plasma membrane [Matsui et al., 1998]. Conceivably, thapsigargin/carbachol treatments also affected the association of TRPC4 with the actin network and caused redistribution of TRPC4 in response to store-depletion induced by thapsigargin and insertion of TRPC4 on the plasma membrane after carbachol stimulation. Thus, physical association with ERM proteins may be one of the reasons that CCE is dependent on the reversible redistribution of actin and is blocked by phosphatase inhibitors. However, although TRPC3 activity was also blocked by preventing actin redistribution, TRPC3 does not interact with NHERF. It is possible that a different PDZ domain-containing protein or a different mechanism is involved in linking TRPC3 to the actin cytoskeleton. It is also possible that TRPC3 multimerizes with TRPC4 or TRPC5, through which it is linked to NHERF. Nevertheless, although TRPC4 mRNA can be detected in HEK 293 cells by RT-PCR, I have failed to detect the endogenous TRPC4 protein in untransfected HEK cells or the stable cell line that expresses TRPC3. TRPC5 has been shown to be expressed only in neuronal cells [Okada et al., 1998; Philipp et al., 1998]. Therefore, it is still unclear whether TRPC3 and other TRPCs except TRPC4 and

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TRPC5 are also linked to cytoskeleton via interaction with PDZ domains and if they are, how many different kind of PDZ-domain containing proteins are involved.

Unexpected from the secretion-like coupling model, there is a significant amount of TRPC4 on the plasma membrane under resting conditions. Therefore, it is unlikely that every TRPC4 channel will undergo a secretion-like insertion into the plasma membrane upon activation. Some channels may be constitutively expressed on the surface and may also be responsible for the spontaneous activity found for TRPC4 overexpressed in HEK293 cells. Alternatively multiple mechanisms may be involved in activating TRPC4-based channels. Conformational coupling and secretion-like coupling may be two components of the same process. The actin cytoskeleton may be involved in both secretion-like coupling and conformational coupling by participating in vesicular trafficking as well as by facilitating the interaction between IP$_3$ receptors and TRPCs [Clapham, 1995]. The fact that both mechanisms are involved in the activation of TRPC channels further substantiates the idea that TRPCs are components of SOCs.

In summary, I have shown that TRPC4 is bound to the first PDZ domain of a scaffolding protein, NHERF. Through this interaction, TRPC4 is associated with a large number of signaling molecules in a supermolecular complex. The binding with NHERF is required for TRPC4 to be associated with F-actin through interaction with members of ERM proteins (Fig. 42). Moreover, I showed that upon stimulation of store-operated Ca$^{2+}$ entry, the association between TRPC4 and ERMs as well as that between TRPC4 and F-actin
were increased. In parallel with this, the amount of TRPC4 on the plasma membrane was also increased by the stimulation of PLC signaling pathway with a receptor agonist, carbachol. The binding to NHERF is critical for these changes because disruptions of the TRPC-NHERF interaction prevented the association of TRPC4 with ERMs and actin as well as carbachol-stimulated increase of TRPC4 expression on the cell surface. Furthermore, I found that removal of Ca\textsuperscript{2+} from extracellular solution caused a large increase in the F-actin content and the association of TRPC4 with actin. The effect of carbachol and thapsigargin on the TRPC4-actin association was smaller in the presence than in the absence of Ca\textsuperscript{2+}. These suggest that Ca\textsuperscript{2+} influx may promote the dissociation of TRPC4 from actin through a negative feedback mechanism. Overall, the data present here support the secretion-like coupling hypothesis of SOCs and will enrich our understanding of the mechanisms of regulation of TRPC-formed channels as well as native SOCs.

4.4. Future work

I have so far obtained direct physical evidence supporting the secretion-like coupling hypothesis. However, the physiological significance of TRPC4’s interaction with NHERF and the consequent association with ERMs and F-actin need to be examined using functional assays. The C-terminal ‘TTRL’-deleted TRPC4 construct will be useful for these test. I have established stably transfected HEK293 cell lines that express either the wild type TRPC4 or the C-terminal ‘TTRL’-deleted TRPC4. Immunostaining of nonpermeabilized cells showed that both forms of TRPC4 are expressed on the plasma
membrane. Ionic currents have been successfully recorded for the wild type as well as the C-terminal 'TTRL'-deleted TRPC4 channel by patch clamp recording (unpublished data). Future work will focus on the detailed characterization of currents carried by the wild type and the mutant TRPC4. Higher constitutive activity or slower inactivation may be anticipated for the mutant channel as compared to the wild type since trafficking is involved in both exocytotic insertion of the channel to plasma membrane and endocytotic retrieval, which presumably could be an essential step of channel inactivation.
Figure 42. Mechanism of TRPC4 association with actin. The C-terminus of TRPC4 binds to the first PDZ domain of NHERF. The last 3 residues of TRPC4, 'TRL', determines the specificity. The C-terminus of NHERF binds to the N-terminus of an activated ERM, which also binds to actin at its C-terminus. The activation of ERMs involves phosphorylation of a C-terminal threonine residue and the conversion from intramolecular to intermolecular interactions.
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