# INVITED SPEAKERS
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# ORAL COMMUNICATIONS

# HOW $Ca^{2+}$ ATPase PUMPS IONS ACROSS THE SARCOPLASMIC RETICULUM MEMBRANE

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$Ca^{2+}$ ATPase of skeletal muscle sarcoplasmic reticulum (SERCA1a) is an integral membrane protein of 110K and the best characterised member of the P-type (or E1/E2-type) ion translocating ATPases. It consists of 10 transmembrane helices (M1-M10), 3 cytoplasmic domains (A, actuator; N, nucleotide binding; P, phosphorylation) and small lumenal loops. According to the classical E1/E2 theory, transmembrane $Ca^{2+}$-binding sites have high affinity and face the cytoplasm in E1; in E2, the binding sites have low affinity and face the lumen of the sarcoplasmic reticulum (SR). Actual transfer of bound $Ca^{2+}$ is thought to take place between the two phosphorylated intermediates, E1P and E2P. At the same time, 2 or 3 protons are countertransported from the lumen of SR to the cytoplasm, despite that the SR membrane is leaky to protons.

We have determined the crystal structures of this ATPase in 8 different states that cover nearly the entire reaction cycle (reviewed recently: Toyoshima, 2008), and also carried out all-atom molecular dynamics simulations.

These analyses show that ion pumps use large rearrangements of cytoplasmic domains to move transmembrane gates of ion pathway, and that ATP, phosphate, $Ca^{2+}$ and $Mg^{2+}$ are the principal modifiers of the domain interfaces. Large domain movements are necessary because ion pumps suffer from thermal fluctuations yet utilise thermal energy effectively for translocating ions. During the transition from E1P to E2P, the phosphoryl group itself moves only 1 Å. Yet this small movement is transmitted across 50 Å distance and causes large rearrangements of transmembrane helices. We now begin to understand why the structure of the $Ca^{2+}$ ATPase has to be so to achieve such an amazing work.

Toyoshima (2008) Arch. Biochem. Biophys. 476, 3-11.

Movies showing the structural changes during the reaction cycle can be downloaded from my home page (http://www.iam.u-tokyo.ac.jp/StrBiol/resource/res.html).

**Keywords: SERCA, catalytic cycle, sarcoplasmic reticulum, X-ray structural analysis**

# T-TYPE $Ca^{2+}$ CHANNELS IN HEALTH AND DISEASE

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$Ca^{2+}$ channels play pivotal roles in physiology, including how our brain works and our heart beats. Twenty-five years after the discovery of low voltage-activated (LVA) / T-type $Ca^{2+}$ currents in neurons and ten years after the cloning of the $Ca_v3$ subunits of T-type $Ca^{2+}$ channels, the recent discoveries confirm that T-channels contribute to a wide variety of physiological and disease states, including thalamocortical rhythms in sleep, hormone secretion, heartbeat, epilepsy, pain processing and cancer. To date however, knowledge about active domains in T-channel proteins, possible partners, and modulation pathways is still very limited. A quick overview of some recent findings obtained in our laboratory supporting the modulatory effects of zinc, bioactive lipids and protein kinases will be discussed in this presentation.

Novel information about T-channel modulation can also be gained from the study of diseased channels. Single nucleotide polymorphisms (SNPs)/mutations in the genes coding $Ca_v3$ subunits are linked to human diseases: childhood absence epilepsy (CAE: $Ca_v3.2$), juvenile myoclonic epilepsy (JME: $Ca_v3.1$) and autism spectrum disease (ASD: $Ca_v3.2$). The intracellular loop between domain I and II (I-II loop) of $Ca_v3.2$ is a “hot-spot” of CAE mutations. These mutations lead to significant increase in surface expression of $Ca_v3.2$ channels and further structure-function analysis revealed that the central region of the I-II loop plays a critical role in the surface expression of $Ca_v3.2$ channels (Vitko et al., 2007).

Overall, these studies reveal novel cellular and molecular paradigms of the altered activity of T-channels and foster novel interest in these channels as drug targets in diseases.

Vitko et al. (2007) J. Neurosci. 27, 322-330.

**Keywords: T-type $Ca^{2+}$ channel, channelopathy, epilepsy**

# VOLTAGE-GATED CALCIUM CHANNELS AND PAIN

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The transmission of pain signals in primary afferent dorsal root ganglion neurons critically relies on voltage-gated calcium channels. T-type channels regulate the excitability and firing properties of these neurons, whereas N-type channels are essential mediators of synaptic transmission between primary afferent neurons and the spinothalamic tract.

Inhibition of N-type calcium channels by pharmacological means - either directly via channel blockers, or indirectly via opioid receptors - therefore mediates analgesia. Certain G protein-coupled receptors form signalling complexes with these channels, thereby fine tuning their regulation and providing for means for receptor-mediated channel trafficking. Channel activity can also be modified by alternate splicing events which allow for specific alterations of N-type channel function in pain sensing neurons.

These considerations underscore the immense complexity of N-type calcium channel regulation in the pain pathway, and may provide for novel means of therapeutic intervention.

**Keywords: N-type $Ca^{2+}$ channels, pain, GPCR**

# CALCIUM CHANNELS AND MIGRAINE

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Migraine is a common disabling brain disorder. A subtype of migraine with aura (familial hemiplegic migraine type 1: FHM1) is caused by mutations in $Ca_v2.1$ (P/Q-type) $Ca^{2+}$ channels. FHM1 mutations shift channel activation to lower voltages and increase $Ca^{2+}$ influx through single recombinant human $Ca_v2.1$ channels.

The analysis of the P/Q $Ca^{2+}$ current in cerebellar, cortical and trigeminal ganglion neurons of knockin (KI) mice carrying a FHM1 mutation revealed neuron-specific gain-of function effect of the mutation, larger in central than peripheral neurons. The KI mice showed a reduced threshold for and increased velocity of cortical spreading depression (CSD), the phenomenon that underlies migraine aura and may activate migraine headache mechanisms. To investigate the mechanisms of CSD facilitation, we studied neurotransmission at synapses of cortical pyramidal cells in microculture and in connected pairs of pyramidal cells and fast-spiking interneurons in acute slices.

We show increased strength of excitatory neurotransmission due to increased action potential-evoked $Ca^{2+}$ influx through synaptic $Ca_v2.1$ channels and increased probability of glutamate release at pyramidal cell synapses of FHM1 KI mice. At the same synapses, short-term depression during trains of action potentials was enhanced. There was no evidence of homeostatic compensatory mechanisms at excitatory synapses onto pyramidal cells. To investigate possible alterations of the cortical excitation-inhibition balance in FHM1, we studied neurotransmission between fast-spiking interneurons and pyramidal cells. At this inhibitory synapse the strength of neurotransmission was unaltered in KI mice.

Our findings may explain CDS facilitation in FHM1 mice and point to cortical network hyperexcitability as the basis for increased CSD propensity and abnormal processing of sensory information in migraine.

**Keywords: P/Q-type $Ca^{2+}$ channel, hemiplegic migraine, neurons, channelopathy**

# A MOLECULAR MECHANISM FOR CRAC CHANNEL ACTIVATION

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The $Ca^{2+}$-release-activated $Ca^{2+}$ (CRAC) channel is a prototypic store-operated $Ca^{2+}$ channel (SOC) that generates sustained signals essential for a range of cell functions, including antigen-stimulated T lymphocyte activation and proliferation. The means by which the loss of ER $Ca^{2+}$ triggers the opening of SOCs in the plasma membrane has been the longstanding mystery underlying store-operated $Ca^{2+}$ entry (SOCE).

The identification of STIM1 as the ER $Ca^{2+}$ sensor and Orai1 as the pore-lining subunit of the CRAC channel has now made it possible to visualize and manipulate the sequence of events that links store depletion to CRAC channel opening. Following store depletion, STIM1 oligomerizes and moves from locations throughout the ER to accumulate in ER-plasma membrane (ER-PM) junctions, a subset of ER positioned 10-25 nm from the plasma membrane. Simultaneously, Orai1 gathers at discrete sites in the plasma membrane directly opposite STIM1, resulting in local CRAC channel activation and $Ca^{2+}$ entry. The $[Ca^{2+}]_{ER}$-response relation for the CRAC channel is a highly cooperative function which is determined by the similarly steep dependence of STIM1 redistribution on $[Ca^{2+}]_{ER}$. These results suggest that STIM1 must first oligomerize in order to accumulate at ER-PM junctions. To assess the role of STIM1 oligomerization in SOCE, we replaced the luminal $Ca^{2+}$-sensing domain of STIM1 with the rapamycin-binding proteins FRB or FKBP. Upon addition of a rapamycin analog, the fusion proteins oligomerize, accumulate at ER-PM junctions and activate CRAC channels without depleting $Ca^{2+}$ from the ER.

Thus, STIM1 oligomerization triggers the redistribution of the CRAC channel and its sensor, leading to the self-organized assembly of active STIM1-Orai1 complexes at ER-plasma membrane junctions. In this way, oligomerization of STIM1 serves as the critical transduction event that couples $Ca^{2+}$-store depletion into the graded activation of $Ca^{2+}$ entry through CRAC channels.

**Keywords: store-operated $Ca^{2+}$ entry, Orai1, STIM1**

# TRPC CHANNELS AND PULMONARY HYPOXIA

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Mutations in TRP channels have been identified as the molecular cause of several human diseases. Apart from their obvious role in pathophysiology, they are centrally involved in the regulation of essential physiological processes. Regional alveolar hypoxia causes local vasoconstriction in the lung, shifting blood flow from hypoxic to normoxic areas thereby maintaining gas exchange. This mechanism is known as hypoxic pulmonary vasoconstriction (HPV).

Using TRPC6-deficient mice, we show that this channel is a key regulator of acute HPV as acute HPV was absent in TRPC6$^{-/-}$ mice. Induction of regional hypoventilation resulted in severe arterial hypoxemia in TRPC6$^{-/-}$ but not in wild-type (WT) mice. This effect was mirrored by a lack of hypoxia-induced cation-influx and -currents in smooth muscle cells from precapillary pulmonary arteries (PASMC) of TRPC6$^{-/-}$ mice. In both WT and TRPC6$^{-/-}$ PASMC hypoxia caused diacylglycerol (DAG) accumulation. We conclude that TRPC6 plays a unique and indispensable role in acute hypoxic pulmonary vasoconstriction.

Apart from pulmonary artery smooth muscle cells, lung endothelial cells are also affected by hypoxia. Lung endothelial damage is a characteristic morphological feature of ischemia-reperfusion (I/R) injury, although the molecular steps involved in the loss of endothelial integrity and subsequent invasion of immune cells are still poorly understood. Isolated lungs from TRPC6-deficient mice are resistant to I/R injury, while lungs from wild-type mice show an increased capillary filtration coefficient (Kfc) resulting in pulmonary edema formation in reperfused areas. Primary isolated endothelial cells from TRPC6-deficient lungs show a reduced hypoxia-induced $Ca^{2+}$ influx and actin stress-fiber formation, as well as a decreased cellular shape change compared to cells from wild-type lungs.

Therefore, TRPC6 may represent a promising pharmacological target for the control of pulmononary hemodynamics and gas exchange.

**Keywords: TRPC6, ischemia, vascular myocytes, endothelium, lung**

# RNA EDITING OF Orai1 IS A KEY TO DIFFERENT $Ca^{2+}$ SELECTIVITY OF STORE-OPERATED CHANNELS IN EXCITABLE AND NON-EXCITABLE CELLS

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Store-operated $Ca^{2+}$ entry (SOCE) is known to be mediated by store-operated channels (SOCs) that have different $Ca^{2+}$ selectivity. While Orai1 is thought to encode $Ca^{2+}$-selective SOC (CRAC) in nonexcitable cells, the molecular nature and the reasons for a poor cation selectivity of SOCs in other cell types (including SMC) remain a mystery. Here we present first evidence that Orai1 may encode all SOC channels, and variations in selectivity can result from post-transcriptional modification of Orai1, with ADAR-mediated RNA editing being a key to this process.

Patch-clamp, $Ca^{2+}$ imaging and molecular approaches were used to study $Ca^{2+}$-selective CRAC in RBL-2H3 cells and nonselective cation SOC (cat-SOC) in SMC. We discovered that: 1) siRNA knock down of Orai1 impairs whole-cell SOC currents in both, RBL and SMC cells; 2) ADAR1 is highly expressed in SMC, but not RBL cells; 3) overexpression of ADAR1 in RBL results in transformation of inwardly rectifying $I_{CRAC}$ into a linear current, and appearance of significant $Mn^{2+}$ influx through SOCE pathway; 4) siRNA knock down of endogenous ADAR1 in SMC results in disappearance of significant $Mn^{2+}$ influx, and appearance of inwardly rectifying $I_{CRAC}$ component of the whole-cell current. Thus, expression and functional activity of ADAR1 can change the selectivity of SOC channels.

To confirm that Orai1 indeed is a target for RNA editing that can change its selectivity, we created an Orai1 mutant with a single point mutation that mimics editing event. Expression of this edited Orai1 in RBL cells created a fully functional nonselective cation channel that was undistinguishable from cat-SOC in SMC.

These breakthrough findings resolve the long lasting controversy about the relationship between SOC channels with different cation selectivity, and demonstrate how one gene (Orai1) can encode SOCs with totally different $Ca^{2+}$ selectivity, with ADAR-dependent RNA editing adjusting it to the specific needs of different cell types.

**Keywords: store-operated $Ca^{2+}$ entry, Orai1, ADAR1**

# STORE-OPERATED CALCIUM ENTRY IS SUPPRESSED DURING MITOSIS DUE TO PHOSPHORYLATION OF THE ENDOPLASMIC RETICULUM CALCIUM SENSOR STIM1

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Depletion of endoplasmic reticulum (ER) $Ca^{2+}$ stores activates $Ca^{2+}$ influx via plasma membrane (PM) $Ca^{2+}$ channels in a process known as store-operated $Ca^{2+}$ entry (SOCE). SOCE involves Orai $Ca^{2+}$ influx channels and the STIM1 ER $Ca^{2+}$ sensor. When $Ca^{2+}$ stores are full, STIM1 is localized in tubular structures throughout the ER membrane; in contrast, when $Ca^{2+}$ stores are depleted, STIM1 rearranges into punctate structures near the PM where it activates Orai channels. Thus, the SOCE function of STIM1 depends on transitions between distinct localization states, and we have demonstrated that STIM1 localization and function rely on microtubules (MT). This led us to consider what happens to SOCE during mitosis, when the cell and the MT cytoskeleton in particular undergo significant morphological changes.

We found that in mitotic HeLa cells, an enhanced yellow fluorescent protein-tagged STIM1 (eYFP-STIM1) did not localize to MTs and did not rearrange into near-PM puncta in response to $Ca^{2+}$ store depletion. Consistent with this, $Ca^{2+}$ store depletion did not activate SOCE in mitotic cells. Mitosis-specific phosphorylation of STIM1 may contribute to SOCE suppression, because the phospho-specific MPM-2 antibody recognized eYFP-STIM1 immunoprecipitated from mitotic but not interphase cells. MPM-2 recognizes phosphorylated serine or threonine followed by proline, a motif found in the consensus sites for mitotic kinases such as cyclin-dependent kinase 1 (cdk-1) and mitogen-activated protein kinase (MAPK). STIM1 contains one cdk-1 and three MAPK consensus sites.

We are currently performing site-directed mutagenesis of these sites and mass spectrometry analysis to determine whether STIM1 phosphorylation underlies SOCE suppression during mitosis and to identify the kinase responsible. Suppression of SOCE during mitosis may be an important signaling event, since mitotic processes such as chromosome separation and cytokinesis are exquisitely sensitive to small changes in cytoplasmic $Ca^{2+}$.

**Keywords: STIM1, mitosis, MPM2-phosphorylation, store-operated $Ca^{2+}$ entry**

# RAGE, DIABETES AND INFLAMMATION RESPONSES

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Advanced glycation endproducts (AGEs) form when the carbonyl group of glucose directly condenses with free amino groups on proteins, particularly on accessible lysines or arginines. This process leads to the formation of a broad range of species collectively termed AGEs. It is the heterogeneity of AGEs that likely reflects the diverse situations in which they are now known to form. Beyond diabetes and natural aging, AGEs form in advanced renal failure and atherosclerosis, settings in which diabetic subjects are already highly vulnerable. Furthermore, complications arising from atherosclerosis, characterized by superimposed hypoxia and ischemia/reperfusion (I/R) injury, are potent and rapid generators of AGEs, both in the presence and absence of diabetes.

The principal cell surface signal transduction receptor for AGEs is the Receptor for AGE, or RAGE. Importantly, the biology of RAGE is complex. In addition to AGEs, RAGE is a signaling receptor for non-AGE ligands. Certain members of the S100/calgranulin family of pro-inflammatory and migration-stimulating molecules bind RAGE, activate signaling, and modulate cellular gene expression and function. For example, S100A12 and S100B have been identified in multiple cell types as RAGE ligands. High Mobility Group Box-1 (HMGB1), in homeostasis, is a nuclear protein. In stress, however, HMGB1 may migrate to the leading edge of activated cells or be fully released into the microenvironment. In such milieu, HMGB1 may bind to and activate RAGE, thereby stimulating MAP kinase activation and upregulation of matrix metalloproteinase (MMP) protein and activity.

RAGE is expressed on multiple cell types – from vascular and inflammatory cells – to neurons (central and peripheral nervous systems), glomerular epithelial cells (podocytes), and cardiomyocytes – all cells highly susceptible to complications in diabetes.

Experimental evidence using antagonists of the ligand-RAGE axis, such as soluble RAGE and anti-RAGE IgG, as well as genetically-modified mice (RAGE null and transgenic mice in which signal transduction is deficient in specific cell types) reveal that both vascular and inflammatory stresses, at least in part via RAGE, contribute integrally to the complications of diabetes and inflammatory responses.

In this presentation, the role of RAGE in vascular and inflammatory cell stress will be discussed.

**Keywords: RAGE, diabetes mellitus, inflammation**

# STRUCTURE OF RAGE AND MECHANISM OF ACTIVATION BY S100 PROTEINS

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The S100 family is a specialized group of tissue- and cell-type specific EF-hand $Ca^{2+}$-binding proteins found only in humans and other vertebrate animals. S100s are distinguished from all other EF-hand proteins by their ability to be secreted into the extracellular space. Once outside the cell, specific S100 proteins have been shown to stimulate neurite outgrowth, participate in the innate immune response and activate cell surface receptors. The majority of studies of S100 proteins have investigated the intracellular functions of S100 proteins; considerably less is known about how S100 proteins evoke biological activity in the extracellular milieu.

S100 protein activation of RAGE is a key pro-inflammatory signal in acute and chronic inflammation. RAGE signaling leads to increases in reactive oxygen species and the induction of transcription of proinflammatory genes, such as the transcription factor NF-κB, the downstream effects of which result in vascular tissue injuries of importance in diabetes, chronic inflammatory disorders and the development of certain cancers.

A combination of X-ray crystallography, NMR spectroscopy, biochemical and structural modeling techniques has been used to obtain insights into the structure of RAGE and the mechanism for activation by S100 proteins. RAGE is a member of a class of receptors that initiate a signal through direct interaction with kinases.

The most likely models for RAGE activation, which involve aggregation of receptors at the cell surface to co-localize enough receptors to activate the signaling cascade, will be discussed in light of the structural properties of S100 proteins.

**Keywords: S100 proteins, RAGE, inflammation, computational modelling, X-ray structural analysis**

# INTERACTION OF RAGE WITH S100 PROTEINS: AN UPDATE

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Ten out of 21 S100 proteins are currently known to bind to Receptor for Advanced Glycation End products (RAGE) and the interaction between S100 and RAGE may contribute to the development and progression of several important human diseases. This review focuses on the members of the S100 protein family that have been shown to interact with RAGE in cell-based assays or in vitro, and their binding mode to RAGE. Despite the high degree of sequence and structural homology among S100 proteins their interactions with RAGE appear to be surprisingly diverse. For S100B and S100A12 detailed information about the RAGE domain most critical for ligand binding have been identified and models of ligand-induced RAGE multimerization have been proposed. For the other S100 proteins much less information is available. We will present and discuss here our latest binding data about the calcium-dependent interaction of RAGE with S100A1,S100A2, S100A4 and S100A6.

**Keywords: RAGE, S100 proteins**

# CALMYRIN 2, A NOVEL EF-HAND $Ca^{2+}$-BINDING PROTEIN, IS INDUCED IN NEURONS BY NMDA RECEPTOR ACTIVATION

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Calmyrin 2 is one of four human genes encoding members of the EF-hand $Ca^{2+}$-binding calmyrin (CaMy) protein family. The only characterized protein of the CaMy family is CaMy1, implicated in Alzheimer disease.

We cloned CaMy2 from rat brain, demonstrated its $Ca^{2+}$-sensor properties and report the analysis of CaMy2 expression in rat brain. CaMy2 is an extended monomer which changes conformation upon $Ca^{2+}$ binding. CaMy2 transcript and protein are preferentially expressed in neurons of the hippocampus and cortex. Moreover, CaMy2 expression in rat brain was significantly increased upon metrazol shock treatment. Further studies on CaMy2 expression were performed in primary culture of hippocampal neurons.

We found that mRNA and CaMy2 protein expression is induced by NMDA stimulation and that this induction can be blocked by $Ca^{2+}$ chelator BAPTA or specific inhibitors of NMDA receptor. Moreover, we demonstrate that NMDA-induced CaMy2 expression regulation is mediated by protein kinase C and ERK1/2. Double-labelling studies in NMDA-treated neurons revealed that CaMy2 is colocalized in neurites and in the Golgi apparatus. In contrast, NMDA does not induce CaMy1, and CaMy1 is present in neurons mainly in the nucleus.

Our results point to CaMy2 involvement in $Ca^{2+}$ signaling mediated by NMDA receptor activation. This report indicates also that localization and putative functions of CaMy2 in neurons differentiate it from its closest homolog, CaMy1.

Supported by the Polish grants PBZ-KBN-124/P05/2004 and N301 109 32/3854.

**Keywords: EF-hand, neurons, calmyrin 2, ionotropic glutamate receptor**

# ANALYSIS OF NCS-1 INTERACTIONS USING TRANSGENIC MICE EXPRESSING NCS-1-EGFP FUSION PROTEIN IN FOREBRAIN

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NCS-1 is a member of the neuronal calcium sensor family of EF-hand containing calcium-binding proteins. NCS-1 has been implicated in the modulation of synaptic efficacy and secretion. In yeast the NCS-1 orthologue Frq1 functions as regulatory subunit of phosphatidylinositol 4-kinase Pik1. Pik1-activity affects Golgi function and vesicular trafficking. The function of mammalian NCS-1, however, is controversial. Using mostly in vitro assays with transiently transfected cells mammalian NCS-1 has been proposed to have diverse functions and molecular roles, for example in regulation of ion channels and enzymatic activities and/or in receptor endocytosis and in neurite outgrowth.

We were interested to characterise potential protein interaction partners of NCS-1 in vivo. Towards this goal we generated transgenic mice expressing GFP-tagged NCS-1 (C2a-NCS1E) under control of the forebrain-specific promoter of the α subunit of $Ca^{2+}$-calmodulin-dependent protein kinase II (CaMKIIα). NCS-1-EGFP containing protein complexes were isolated from forebrain lysates by a combination of protein-chromatography and anti-GFP co-immunoprecipitation. For control, we used lysates from mice expressing EGFP under CaMKIIα-promoter control. Immunoprecipitated proteins were separated by polyacrylamide gel electrophoresis. Sequences of specific proteins which were only present in NCS-1-EGFP immunoprecipitates were obtained by subsequent liquid chromatography-tandem mass spectrometry. We identified several candidate proteins.

One candidate protein (actin-related protein 1, ARP1) was investigated further. We show that NCS-1-EGFP and ARP1 interact in a $Ca^{2+}$-dependent manner. ARP1, a subunit of the dynactin protein complex, is involved in vesicle endocytosis. We propose that NCS-1 in vivo plays a role in this process.

**Keywords: neuronal $Ca^{2+}$ sensors, endocytosis**

# DEVELOPMENTAL REGULATION OF $Ca^{2+}$ - SECRETION COUPLING AT A LARGE CNS SYNAPSE

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After synapses form during brain development, their function is optimized with further postnatal development, and these developmental refinements likely include a tighter $Ca^{2+}$ channel - vesicle co-localization (Fedchyshyn and Wang, 2005). It is unknown, however, whether the intracellular $Ca^{2+}$ sensitivity of vesicle fusion could change during development. At the calyx of Held, a large excitatory synapse of the auditory system, direct measurements by presynaptic $Ca^{2+}$ uncaging have shown a surprisingly high intracellular $Ca^{2+}$ sensitivity of vesicle fusion (Bollmann et al., 2000; Schneggenburger and Neher, 2000), but these measurements have so far been limited to calyces of Held of young animals (P8 - P10).

Here, we studied mechanisms of $Ca^{2+}$ - secretion coupling at the calyx of Held synapse at two developmental stages right before (P8-9), and after the onset of hearing (P12 - P15). We found an ~2-fold leftward shift in the relationship between EPSC amplitude and presynaptic $Ca^{2+}$ current-charge ($Q_{Ca}$), indicating an enhanced efficiency of brief $Ca^{2+}$ currents in evoking release, but the steepness of the EPSC - $Q_{Ca}$ relationship was high both before and after hearing onset (power of ~3.5). In contrast to the leftward shift of the EPSC - $Q_{Ca}$ curve, the intracellular $Ca^{2+}$ sensitivity of vesicle fusion, as measured in presynaptic $Ca^{2+}$ uncaging experiments, was unchanged or even slightly rightward-shifted after hearing onset.

These findings show that an increased spatial co-localization between readily-releasable vesicles and $Ca^{2+}$ channels must cause the enhanced efficiency of brief $Ca^{2+}$ currents in evoking release. Nevertheless, the high cooperativity of brief $Ca^{2+}$ currents in evoking release at both ages indicates the persistence of multiple $Ca^{2+}$ channels in the release control of a given readily-releasable vesicle.

Bollmann et al. (2000) Science 289, 953-957.
Fedchyshyn and Wang (2005) J. Neuroscience 25, 4131-4140.
Schneggenburger and Neher (2000) Nature 406, 889-893.

**Keywords: auditory synapse, $Ca^{2+}$-secretion coupling**

# DOWNSIZING OF AMPA MEDIATED mEPSCS AND REDUCTION OF GABAergic SYNAPSES IN CYSTEINE STRING PROTEIN-α DEFICIENT HIPPOCAMPAL NEURONS

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Cysteine String Protein-α (CSP-α) is a synaptic vesicle protein that was originally proposed to play a regulatory role on voltage-dependent $Ca^{2+}$ channels and on the machinery of $Ca^{2+}$-dependent exocytosis (Evans et al., 2003). Interestingly, it has been found that CSP-α prevents nerve terminal degeneration (Fernández Chacón et al., 2004; Schmitz et al., 2006) in cooperation with alpha-synuclein (Chandra et al., 2005). Probably, CSP-α action is most required when continuous synaptic activity increases but detailed mechanisms are not well known.

We have analyzed synaptic transmission on hippocampal cultures from knock-out mice lacking CSP-α. We have found that CSP-α is not a major player to execute excitatory neurotransmission in autaptic hippocampal cultures. In contrast, in long-term neuronal cultures forming networks of excitatory and inhibitory neurons, CSP-α is required to maintain synapse number. Unexpectedly, the frequency of excitatory AMPA mEPSC is normal but the amplitude progressively decreases in parallel with culture aging. Those changes resemble the typical synaptic scaling of homeostatic plasticity triggered by blockers of inhibitory synapses (Turrigiano et al., 1998). Consistently, we discovered that the number of GABAergic synapses was dramatically reduced in the cultures lacking CSP-α. Furthermore, typically active parvalbumin interneurons almost disappeared while putatively less active calretinin labelled interneurons did not change in number.

Those results reveal a preferential need for CSP-α to maintain GABAergic synapses and, probably, interneurons that are typically more active. In addition, our results enlighten that homeostatic plasticity might also emerge as a general mechanism to stabilize network activity during neurodegeneration.

Chandra et al. (2005) Cell 123, 383-396.
Evans et al. (2003) Traffic 4, 653-659.
Fernández-Chacón et al. (2004) Neuron 42, 237-251.
Schmitz et al. (2006) Proc. Natl. Acad. Sci. USA 103, 2926-2931.
Turrigiano et al. (1998) Nature 391, 892-896.

Supported by: Spanish Ministry of Education and Science (BFU2005-08130, BFU2007-66008).

**Keywords: synapse, neural circuits, neurodegeneration, cysteine string protein-α**

# $Ca^{2+}$ REGULATION OF HAIR CELL EXOCYTOSIS

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Sound coding at hair cell ribbon synapses is tightly regulated by $Ca^{2+}$. Presynaptic $Ca^{2+}$ influx occurs at ≈ 300 nm sized clusters of ≈ 80 $Ca_v1.3$ channels, whose trafficking and function depend on $Ca_v\beta2$. Both, $Ca_v\alpha1.3$ and $Ca_v\beta2$ subunits are essential for hearing.

The $Ca_v1.3$ channels of hair cells show little little $Ca^{2+}$-dependent inactivation (CDI) due to efficient antagonism of calmodulin-mediated CDI by $Ca^{2+}$-binding proteins. Lack of all three cytosolic $Ca^{2+}$ binding proteins parvalbumin-α, calbindin 28-k and calretinin in hair cells speeds up CDI and augments sustained exocytosis while leaving fast exocytosis unchanged. Synaptic $Ca^{2+}$ influx cause the build-up of $Ca^{2+}$ domains within few milliseconds that reach low micromolar average concentrations and submicrometer size. The synaptic $Ca^{2+}$ signal is affected by cytosolic $Ca^{2+}$ buffering but does not involve $Ca^{2+}$-induced $Ca^{2+}$ release. Analysis of the apparent $Ca^{2+}$ dependence of exocytosis during $Ca^{2+}$ influx indicates that these $Ca^{2+}$ microdomains may be composed of smaller nanodomains arising around one or few $Ca_v1.3$ channels.

While changing $Ca^{2+}$ influx by manipulation of single channel current revealed the known non-linear $Ca^{2+}$ dependence, it was near linear upon manipulation of the open probability. The molecular basis of $Ca^{2+}$-dependent exocytosis in hair cells is far from being understood. The classical neuronal SNARE proteins seem not to be involved as indicated by expression analysis and botulinum toxin experiments. The synaptic vesicle C2-domain protein otoferlin is essential for exocytosis and hearing, but its exact function remains to be established.

In summary, $Ca^{2+}$-regulated transmitter release at hair cell ribbon synapses is - in many ways - specialized for temporally precise, reliable coding of graded stimuli.

**Keywords: hair cell, synapse, $Ca^{2+}$-binding proteins, exocytosis, $Ca^{2+}$ microdomain**

# THE COFFIN-LOWRY SYNDROME-ASSOCIATED PROTEIN RSK2 IS IMPLICATED IN CALCIUM-REGULATED EXOCYTOSIS THROUGH THE REGULATION OF PLD1

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Exocytosis of neurotransmitters and hormones occurs through the fusion of secretory vesicles with the plasma membrane. This highly regulated process involves key proteins, such as SNAREs, and specific lipids at the site of membrane fusion. Phospholipase D (PLD) has recently emerged as a promoter of membrane fusion in various exocytotic events potentially by providing fusogenic cone-shaped phosphatidic acid.

We show here that PLD1 is regulated by ribosomal S6 kinase 2 (RSK2)-dependent phosphorylation. RSK2 is activated by a high $K^+$-induced rise in cytosolic calcium. Expression of inactive RSK2 mutants or selective knockdown of endogenous RSK2 dramatically affects the exocytotic response in neuroendocrine chromaffin and PC12 cells. RSK2 physically interacts with and stimulates PLD activity through the phosphorylation of Thr-147 in the PLD1 amino-terminal phox homology domain.

Expression of PLD1 phosphomimetic mutants fully restores secretion in cells depleted of RSK2, suggesting that RSK2 is a critical upstream signaling element in the activation of PLD1 to produce the lipids required for exocytosis.

We propose that PLD-related defects in neuronal and endocrine activities could contribute to the effect observed after the loss-of-function mutations in RSK2 that lead to Coffin-Lowry syndrome, an X-linked form of growth and mental retardation.

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**Keywords: exocytosis, phospholipase D1, RSK2, Coffin-Lowry syndrome**

# WIDELY DIVERSE MODES AND SITES OF $Ca^{2+}$ SIGNALING AND SIGNAL DOWNREGULATION IN THE CILIATED PROTOZOAN, PARAMECIUM TETRAURELIA

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The focus of our group is $Ca^{2+}$-dependency of exocytosis of dense-core secretory vesicles (trichocysts) in Paramecium cells, as well as ciliary beat regulation.

We face the problem of highly mobile cells, refusing the uptake of fluorochrome esters, and rapid fluorochrome sequestration. We injected fluorochromes, and combined stimulation eventually with rapid confocal $Ca^{2+}$ imaging. We also measured $Ca^{2+}$/calmodulin-activated currents over the cell membrane to monitor subplasmalemmal $Ca^{2+}$ currents. This was complemented by quench-flow stimulation, from 30 ms on, followed by structural EM analysis and a protocol retaining $Ca^{2+}$ for energy-dispersive x-ray microanalysis. All this occurred within ~30 ms, slightly below the apparent halftime of synchronous exocytosis whose kinetics depends of extracellular $Ca^{2+}$, $[Ca^{2+}]_o$, although the mechanism of $Ca^{2+}$ signaling during exo-endocytosis involves a store-operated $Ca^{2+}$ influx. The stores are the “alveolar sacs”, flat compartments tightly attached to the cell membrane (just like “inner membrane complexes” in related parasites, Toxoplasma and Plasmodium). We have characterized only recently their rather unorthodox $Ca^{2+}$-release channels (Ladenburger et al., 2006).

While the signal sweeps into the cell, it is rapidly bound to the infraciliary lattice, a cortical meshwork containing the $Ca^{2+}$-binding protein, centrin (Sehring et al., 2008). Knock-out cells show its high $Ca^{2+}$-buffering capacity. Further on, $Ca^{2+}$ is downregulated as follows. As $Ca^{2+}$-pumps in the cell membrane and in the cortical stores proved much too slow, we envisaged the osmoregulatory system (ORS, known to release $Ca^{2+}$). Its $H^+$-ATPase can drive an antiport system, as the downregulation of $Ca^{2+}$ can be inhibited by concanamycin A (work in progress). The $IP_3$ receptors we found in the ORS may serve fine-tuning and their expression depends on $[Ca^{2+}]_o$. A number of additional unorthodox $Ca^{2+}$-release channels are currently under investigation.

Ladenburger et al. (2006) J. Cell Sci. 119, 3705-3717.
Sehring et al. (2008) Cell Calcium (in press).

**Keywords: $Ca^{2+}$ signalling, exocytosis, Paramecium, $Ca^{2+}$ release, centrin**

# SECRETORY PATHWAY $Ca^{2+}$, $Mn^{2+}$ ATPases

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The Secretory Pathway Calcium ATPases (SPCA) comprise a subtype of thapsigargin-insensitive calcium pumps that localize to the Golgi and post-Golgi vesicles. There are two human isoforms: SPCA1 is ubiquitously expressed and appears to play a housekeeping role, whereas SPCA2 has a more restricted tissue distribution, including brain, gastrointestinal system and mammary glands, where it is induced in response to physiological and pathophysiological stimuli.

These transporters supply the Golgi lumen with $Ca^{2+}$ and $Mn^{2+}$ ions that are essential for protein sorting, processing and glycosylation. Based on their distribution and properties, the SPCA are implicated in diverse physiological functions, including regulation of desmosomal contacts in keratinocytes, calcium absorption in intestine, milk secretion in mammary gland and manganese detoxification in bile. Haploinsufficiency of SPCA1 leads to an ulcerative skin disorder, Hailey Hailey disease, and homozygous null mice exhibit embryonic lethality.

We describe the use of the yeast S. cerevisiae as a first approach to characterize pump isoforms, to probe the molecular basis of ion selectivity and to evaluate phenomic characteristics related to disease. Ongoing studies in polarized mammalian cells in culture reveal a role for ion-dependent trafficking that may be critical for a role in ion homeostasis and signaling.

**Keywords: Golgi apparatus, manganese, yeast, SPCA**

# THE CONTROL OF SARCOPLASMIC RETICULUM CALCIUM IN THE HEART

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Most of the calcium that activates cardiac contraction comes from the sarcoplasmic reticulum (SR). Calcium release from the SR is a steep function of SR content and it is therefore important that SR content be regulated.

In the first part of this talk I will review how SR calcium content is controlled. Briefly, an increase of SR calcium content results in an increase of the amplitude of the systolic Ca transient. This, in turn, increases $Ca^{2+}$ efflux from the cell (on $Na^{+}$-$Ca^{2+}$ exchange, NCX) and decreases influx (via the L-type $Ca^{2+}$ current) resulting in a decrease of SR calcium content. This mechanism achieves a similar result as do the store-operated channels seen in other tissues. It also modifies the effects of other manoeuvres that alter calcium handling.

In the second part of the talk I will consider the effects of modifying various steps involved in calcium handling and will demonstrate the lack of effect of increasing the opening of the ryanodine receptor during systole.

The final part of the talk will consider the production of arrhythmias resulting from abnormal SR $Ca^{2+}$ release.

**Keywords: cardiomyocytes, sarcoplasmic reticulum, arrhythmia, $Ca^{2+}$ stores**

# LIPID INTERACTIONS WITH $Ca^{2+}$ ATPase

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The activity of the $Ca^{2+}$ ATPase of skeletal muscle sarcoplasmic reticulum, like that of all membrane proteins, is affected by the structures of the lipid molecules that surround it in the membrane, so that proper function of the ATPase requires the membrane to have the appropriate lipid composition.

The lipid that supports the highest rate of hydrolysis of ATP by the $Ca^{2+}$ ATPase is phosphatidylcholine and high concentrations of anionic lipid result in low rates of ATP hydrolysis. However, hydrolysis of ATP by the $Ca^{2+}$ ATPase reconstituted into vesicles of phosphatidylcholine alone results in low levels of accumulation of calcium, and the presence of anionic lipid is required to achieve high levels of accumulation. It is proposed that the presence of anionic lipid results in a reduction in slippage on the $Ca^{2+}$ ATPase, a process in which the phosphorylated intermediate releases calcium on the external rather than the lumenal side of the vesicle.

The effect of anionic lipid on accumulation of calcium is likely to follow from binding of anionic lipid to specific sites on the transmembrane surface of the $Ca^{2+}$ ATPase. Studies with other membrane proteins such as the mechanosensitive channel of large conductance (MscL) have shown the presence of hot-spots for binding anionic lipid, and have shown that binding of anionic lipid to these sites affects channel function. Binding of phospholipids to crevices between transmembrane α-helices, like that occupied by the hydrophobic inhibitor thapsigargin, could also be important for function.

**Keywords: SERCA, phospholipids, sarcoplasmic reticulum, catalytic cycle**

# $Ca^{2+}$ LOADING IN THE ER-MITOCHONDRIA

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Mitochondria rapidly accumulate $Ca^{2+}$ through a low-affinity uptake system (the mitochondrial $Ca^{2+}$ uniporter, MCU) because they are exposed to high [$Ca^{2+}$] microdomains generated by the opening of ER $Ca^{2+}$ channels. These rapid [$Ca^{2+}$] changes stimulate $Ca^{2+}$-sensitive dehydrogenases of the mitochondrial matrix, and hence rapidly upregulate ATP production in stimulated cells. At the same time, $Ca^{2+}$ sensitizes to cell death mediators acting on mitochondria, such as ceramide. In agreement with this notion, we demonstrated that Bcl-2 reduces the state of filling of ER $Ca^{2+}$ stores, and this alteration is effective in reducing the sensitivity to various apoptotic challenges. I will here review the latest data of the lab focusing on:

1) The effect on mitochondrial $Ca^{2+}$ homeostasis of other signalling pathways involved in apoptosis (Akt, FHIT).

2) The signalling route that links oxidative stress to the activation of p66shc, an isoform of a growth factor adapter acting as apoptotic inducer. We demonstrated that PKCβ, activated by the oxidative challenge, induces p66shc phosphorylation, with ensuing alteration of mitochondrial structure and function (Pinton et al., 2007). We also showed that this route is involved in adipose differentiation of muscle-derived precursors, highlighting a novel process of utmost interest in pathophysiological conditions (Aguiari et al., 2008).

3) The molecular elements of the mitochondria-ER $Ca^{2+}$ connection. I will discuss the role of VDAC in rapidly channelling $Ca^{2+}$ through the mitochondrial outer membrane and the specific functions of the various VDAC isoforms.

Aguiari et al. (2008) Proc. Natl. Acad. Sci. USA 105, 1226-1231.
Pinton et al. (2007) Science 315, 659-663.

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**Keywords: mitochondria, $Ca^{2+}$ microdomain, protein kinase C, apoptosis, $Ca^{2+}$ release**

# PRESENILINS FUNCTION AS ENDOPLASMIC RETICULUM CALCIUM LEAK CHANNELS: IMPLICATIONS FOR ALZHEIMER'S DISEASE

**BEZPROZVANNY I.**

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Alzheimer's disease (AD) is a progressive and irreversible neurodegenerative disorder. Mutations in presenilins are responsible for approximately 40% of all early onset familial Alzheimer's disease (FAD) cases in which a genetic cause has been identified. FAD mutations and genetic deletions of presenilins have been linked with calcium ($Ca^{2+}$) signaling abnormalities, but mechanistic basis for these results has not been clearly determined. Presenilins are highly conserved transmembrane proteins that support cleavage of the amyloid precursor protein by gamma-secretase.

In our studies we discovered that in addition to acting as a gamma-secretase, presenilins also function as passive endoplasmic reticulum (ER) $Ca^{2+}$ leak channels. We demonstrate that wild type PS1 and PS2 proteins form low conductance divalent cation-permeable ion channels in planar lipid bilayers. In experiments with PS1/2 double knockout (DKO) mouse embryonic fibroblasts (MEFs) we discovered that presenilins account for ~80% of passive $Ca^{2+}$ leak from the ER. The ER $Ca^{2+}$ leak function of presenilins is independent from their gamma-secretase function. In additional experiments we demonstrated that ER $Ca^{2+}$ leak function of presenilins is impaired by M146V, L166P, A246E, E273A, G384A and P436Q FAD mutations in PS1 and N141I mutation in PS2. In contrast, frontal temporal dementia-associated mutations (L113P, G183V and Rins352) did not appear to affect ER $Ca^{2+}$ leak function of PS1 in our experiments, indicating that the observed effects are disease-specific.

Our data uncover a novel $Ca^{2+}$ signaling function of presenilins and provide support to the potential role of disturbed $Ca^{2+}$ homeostasis in AD pathogenesis. We are in the process of expanding these findings to neuronal system. Our latest findings will be discussed.

Tu et al. (2006) Cell 126, 981-993.
Nelson et al. (2007) J. Clin. Invest. 117, 1230-1239.

**Keywords: Alzheimer's disease, $Ca^{2+}$ leak, presenilins, $Ca^{2+}$ stores**

# IDENTIFICATION OF THE NAADP RECEPTOR AS A NOVEL CLASS OF ION CHANNELS

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Nicotinic acid adenine dinucleotide phosphate (NAADP), was first identified as a potent $Ca^{2+}$-mobilizing messenger in sea urchin eggs, but subsequently shown to play an important role in many mammalian cells and tissues (Lee et al., 2005). One key outstanding question is the identity of the NAADP-regulated $Ca^{2+}$-release channel. Studies in urchin eggs and mammalian cells have suggested, surprisingly, that the NAADP receptor resides on an acidic lysosome-related organelle rather than on the ER, and is biochemically and pharmacologically distinct from either $IP_3$Rs or RyRs (Churchill et al., 2002; Yamasaki et al., 2004; Galione, 2006).

Building on our studies in urchin eggs, where the response to NAADP is particularly marked, we have recently carried out extensive genomic analysis of novel ion channels and identified a strong candidate for the NAADP receptor. The candidate is part of an uncharacterized family of channels distantly related to voltage-gated calcium and sodium channels, exist as multiple isomeric forms, and are distinct from both the $IP_3$R and the RyR. These channels are present in most organisms including humans, mice and urchins and even plants, but their functions in animals remain unknown.

We have raised antibodies against the sea urchin channel proteins and have shown that immunoprecipitated complexes of these proteins show NAADP-binding properties undistinguishable from the native NAADP receptor, suggesting that they are integral components of the receptor. Our findings both confirm the novelty of the NAADP signalling pathway and open up the prospect of a complete molecular characterization of the NAADP receptor, its mechanism of action, and its functional role in the body.

Churchill et al. (2002) Cell 111, 703-708.
Galione (2006) Biochem. Soc. Trans. 34, 922-926.
Lee (2005) J. Biol. Chem. 280, 33693-33696.
Yamasaki et al. (2004) J. Biol. Chem. 279, 7234-7240.

**Keywords: NAADP, NAADP receptor, $Ca^{2+}$ release**

# MEASUREMENT OF CALCIUM SIGNALS DIRECTED TOWARDS CAVEOLAE

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Caveolae are plasma membrane invaginations where many components involved in cell signaling are located. Several caveolar proteins are also responsive to calcium, suggesting that caveolae may function as relay stations where signaling cascades converge to modulate cellular responses. To measure calcium signals in caveolar structures we made a chimeric protein made up of Caveolin-1 (Cav1) fused to Aequorin (Aeq).

The Cav1-Aeq chimera was localized to detergent-resistant membranes in a similar manner to endogenously expressed Caveolin-1. In contrast, the plasma membrane-targeted calcium probe SNAP-Aeq was predominantly detected in detergent-soluble membrane fractions, making this probe ideal for comparing to Cav1-Aeq. The differential distribution of SNAP-Aeq and Cav1-Aeq was also confirmed by immunocytochemistry.

In HeLa cells stimulated with histamine, Cav1-Aeq sensed several fold higher calcium concentrations than SNAP-Aeq or cytosolic Aeq. This suggests that the calcium mobilization was concentrated to caveolae. In support of this, we found that disrupting caveolar structures with methyl-b-cyclodextrin led to an attenuation of the caveolar calcium signals without significantly affecting the cytosolic response.

Caveolae were also found to be involved in store-operated calcium entry. When adding calcium to cells with depleted intracellular stores, the Cav1-Aeq probe detected higher calcium concentrations than SNAP-Aeq, indicating that the calcium entry channels are close to caveolae. The importance of caveolae in calcium entry was corroborated by the observation that STIM1, which binds and activates store operated calcium channels, partially translocated to caveolae when intracellular calcium stores were depleted.

In conclusion, our results show that calcium signals are concentrated to caveolae both during calcium mobilization and calcium entry. The findings also suggest that caveolae are important structures for integrating calcium signals.

**Keywords: caveolae, aequorin, store-operated $Ca^{2+}$-entry**

# THE $Ca^{2+}$-PERMEABLE SV/TPC1 CHANNEL OF PLANTS: ELECTROPHYSIOLOGICAL PROPERTIES AND PHYSIOLOGICAL FUNCTION

**SANDERS D.**

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The central vacuole of many plant cells occupies up to 95% of intracellular volume and is the principal $Ca^{2+}$ store. Studies using aequorin targeted to the vacuolar membrane have established that $Ca^{2+}$ release from the vacuole is an early event associated with some signal transduction processes. Typically, vacuolar diameter is around 50 mm, and since the organelle is also relatively easy to isolate intact from cells, it is readily amenable to patch clamp studies that aim to identify $Ca^{2+}$-permeable channels that might be involved in $Ca^{2+}$ mobilisation. Accordingly, conductances that are activated by $InsP_3$ and cADPR, as well as by membrane voltage, have been identified and characterised, but difficulties in identifying channel-encoding genes has until recently halted progress in ascribing physiological function.

The voltage-gated and $Ca^{2+}$-permeable Slowly-activating Vacuolar (SV) channel was first characterised electrophysiologically over 20 years ago. Using a reverse genetics approach, we have demonstrated that the SV channel is encoded, at least in part, by the Two Pore Channel 1 (TPC1) gene – the sole member of the TPC family in Arabidopsis. We have recently addressed the question of whether the SV channel comprises subunits other than the TPC1 protein. Pull down assays using GST fusions have identified only TPC1 as a candidate interacting protein, consistent with the notion that the SV channel is a dimer of TPC1.

The phenotype of tpc1 knockout mutants demonstrates a role for the TPC1 channel in regulation of germination by the hormone abscisic acid (ABA), as well as in control of stomatal aperture by extracellular $Ca^{2+}$. Furthermore, forward genetic screens have identified TPC1 mutants that are involved in the plant wound response that is mediated by the hormone jasmonate.

Both ABA and jasmonate are known to evoke physiological responses through elevation in cytosolic free $Ca^{2+}$, in accord with TPC1-generated $Ca^{2+}$ signals.

**Keywords: Arabidopsis, vacuole, $Ca^{2+}$ channel, $Ca^{2+}$ release**

# A $Ca^{2+}$ SENSOR / PROTEIN KINASE NETWORK FOR DECODING $Ca^{2+}$ SIGNALS IN PLANTS

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Intracellular release of $Ca^{2+}$ ions belongs to the earliest events in signal perception. $Ca^{2+}$-binding proteins are involved in sensing and relaying these signals to downstream signalling and adaptation responses. Calcineurin B-like proteins (CBLs) represent a group of $Ca^{2+}$-sensor proteins that are closely related to Calcineurin B and Neuronal $Ca^{2+}$ Sensors (NCS). CBLs interact with a group of serine-threonine kinases designated as CBL-interacting protein kinases (CIPKs). In Arabidopsis, 10 CBL-type $Ca^{2+}$ sensor proteins form an interaction network with 25 CIPKs (Batistic and Kudla, 2004). Preferential complex formation of individual CBLs with defined subsets of CIPKs appears to be one of the mechanisms generating the temporal and spatial specificity of $Ca^{2+}$ signals in plant cells.

Reverse genetics and cell biological approaches have begun to unravel the functional principles of this signalling network. I will present results of our characterization of CBL and CIPK loss-of-function mutants and of our investigation of the subcellular localization of all CBLs from Arabidopsis. These studies suggest that CBL/CIPK complexes function predominantly at cellular membranes and can decode $Ca^{2+}$ signals in different compartments. In this context, dual lipid modification by myristoylation and palmitoylation appears to play an important role in determining the plasma membrane targeting of CBL/CIPK complexes (Kim et al., 2007).

Our reverse genetics analyses indicate that alternative complex formation of CIPK-type kinases with different CBLs enables the simultaneous regulation of ion transport processes in different compartments of the plant cell (Batistic et al., 2008). In this way CBL/CIPK complexes contribute to regulating the extrusion of $Na^{+}$ ions in root tissues and in addition regulate the sequestration of $Na^{+}$ in the vacuole in green tissues.

Batistic and Kudla (2004) Planta, 219, 915-924.
Batistic et al. (2008) Plant Cell 20, 1346–1362.
Kim et al. (2007) Plant J. 52, 473-484.

**Keywords: calcineurin B-like proteins, CIPK, Arabidopsis, $Ca^{2+}$ signalling, $Ca^{2+}$-binding proteins**

# PHOSPHORYLATED LONG CHAIN SPHINGOID BASES AS MESSENGERS IN PLANT SIGNALLING

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In mammalian cells sphingosine-1-phosphate (S1P) is a well-established messenger molecule that participates in a wide range of signalling pathways. In plants a similar role is emerging for long chain phosphorylated sphingoid bases. Previous studies revealed that the application of S1P to stomata resulted in an increase in the concentration of guard cell $Ca^{2+}$ and a reduction in stomatal aperture while the use of inhibitors suggested that sphingosine kinase (SPHK) was involved in guard cell ABA - signalling (Ng et al., 2001). The Assmann lab showed that guard-cells formed S1P after treatment with ABA and that in addition to sphingosine, SPHK is able to phosphorylate phytosphingosine, and that phytoS1P can, like S1P, induce stomatal closure (Coursol et al., 2003; 2005).

Our recent work (Worrall et al., in press) is based on manipulating Arabidopsis genes encoding enzymes capable of metabolizing long chain sphingoid bases. We show that SPHK1 encodes an enzyme that phosphorylates sphingosine, phytosphingosine and other sphingoid long-chain bases. The stomata of SPHK1-KD plants were less sensitive, whereas the stomata of SPHK1-OE plants were more sensitive, than wild type to ABA. The rate of germination of SPHK1-KD was enhanced, whereas the converse was true for SPHK1-OE seed. Reducing expression of either the putative Arabidopsis S1P phosphatase (SPPASE) or the DPL1 gene, which encodes an enzyme with S1P lyase activity, individually, had no effect on guard-cell ABA signalling; however, stomatal responses to ABA in SPPASE DPL1 RNAi plants were compromised.

We also found evidence that expression of SPHK1 and SPPASE were coordinately regulated. This might contribute to robustness in guard-cell signalling. The guard cell, germination and other data concerning the role of SPHK in root hair growth suggest that phosphorylated long-chain sphingoid bases such as S1P and phytoS1P are ubiquitous messengers in plants.

Coursol et al. (2003) Nature 423, 651–654.
Coursol et al. (2005) Plant Physiol. 137, 724–737.
Ng et al. (2001) Nature 410, 596–599.
Worrall et al. (2008) Plant J. (in press).

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**Keywords: sphingolipids, sphingosine-1-phosphate, sphingosine kinase, Arabidopsis**

# INVOLVEMENT OF A PUTATIVE GLUTAMATE RECEPTOR IN CRYPTOGEIN-INDUCED PLANT DEFENSE RESPONSES

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Cryptogein, a 10 kDa protein secreted by an oomycete Phytophthora cryptogea, triggers Systemic Acquired Resistance (SAR) and a programmed cell death on tobacco plants. The mode of action of cryptogein has been studied using tobacco cells. After recognition by high affinity binding site located on the plasma membrane, cryptogein induces a calcium influx necessary for the induction of many events including protein phosphorylation, MAPK activation, anion efflux, plasma membrane depolarisation, production of active oxygen species (AOS), nitric oxide (NO) production and free calcium elevations (Lecourieux et al., 2006; Garcia-Brugger et al., 2006).

Here we demonstrate the involvement of a putative plant homolog of neuronal ionotropic glutamate receptors (iGluRs) in calcium signalling in response to cryptogein. Using tobacco cell suspensions expressing aequorin in the cytosol or in the nucleus we first demonstrated that glutamate induces a strong and transient $[Ca^{2+}]_{cyt}$ elevation without $[Ca^{2+}]_{nuc}$ changes. Glutamate-induced $[Ca^{2+}]_{cyt}$ elevation was a result of calcium influx from the extracellular medium and was inhibited by glutamate receptor inhibitors. This data suggest the presence of functional calcium channels of GluRs-type in tobacco. Further, $[Ca^{2+}]_{cyt}$ elevation and calcium influx induced by cryptogein were shown to be partially suppressed by the glutamate receptor inhibitors suggesting that cryptogein could activate a calcium channel of the GluR-type. Interestingly, these inhibitors neither affect nuclear calcium variations induced by cryptogein nor inhibit some of the calcium-dependent cell signalling pathway activated by cryptogein. Thus, we are trying to understand the potential implication of plant homolog GluRs in the activation of the defense mechanism of plants

Garcia-Brugger et al. (2006) MPMI19, 711-724

Lecourieux et al. (2006) New Phytologist 171, 249-269.

**Keywords: glutamate receptor (ionotropic), tobacco cell suspensions, plant defense, $Ca^{2+}$ entry**

# MEMBRANE-INDUCED STRUCTURAL CHANGES IN ANNEXINS

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Annexins are a super-family of soluble $Ca^{2+}$-binding proteins that have been implicated in a number of membrane-related biological events. The functional hallmark of annexins is their ability to reversibly bind to membranes in the presence of $Ca^{2+}$ but recent studies also identified $Ca^{2+}$-independent interaction of certain annexins with cellular membranes.

We used the interaction of annexin B12 with phospholipid vesicles as a model system to investigate the biophysical mechanism by which annexins bind to membranes. A variety of experimental approaches were used including EPR spectroscopy studies of over 200 site-directed spin-labeled derivatives of the protein. These studies showed that annexin B12 bound to the surface of membranes in the presence of $Ca^{2+}$ without undergoing significant changes in backbone fold. All eight loops on the convex face of the protein formed $Ca^{2+}$ bridges between the protein and phospholipid head groups. Both the side chains of loop amino acids and the phospholipid head group region of bilayers became immobilized. These studies provided a biophysical explanation for the popular idea that annexins serve as molecular fences that create or maintain domains of non-homogenous distribution of lipids and/or proteins in cellular membranes.

In the absence of $Ca^{2+}$, annexin B12 bound to membranes either at mildly acid pH or at neutral pH in the presence of small highly curved vesicles or larger vesicles containing lipids with negative intrinsic curvature. $Ca^{2+}$-independent membrane binding caused reversible global “inside-out refolding” in which several helical hairpin regions refolded and formed continuous amphipathic α-helixes. Residues on the hydrophobic face of these helices were in direct contact with the hydrophobic region of membranes. Depending on the experimental conditions, these amphipathic helices were either parallel to the plane of the membrane or perpendicular to the membrane forming transmembrane pores. These studies raise the possibility that annexins act as physiological sensors of cellular membrane curvature or curvature strain.

**Keywords: annexin B12, phospholipids**

# v-Src INDUCED RUFFLING, MACROPINOCYTOSIS, FOCAL ADHESION REMODELLING AND CELLULAR TRANSFORMATION REQUIRE ANNEXIN 2

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Cell transformation by v-Src involves rearrangement of the actin cytoskeleton, disassembly of focal adhesions and the development of anchorage-independent growth. We have discovered that this is dependent on annexin 2, a v-Src substrate and calcium-dependent regulator of actin dynamics.

Using a thermoactivatable mutant of v-Src, we show that at the permissive temperature annexin 2 becomes phosphorylated and co-localises with activated v-Src, FAK, actin and paxillin at the plasma membrane, ruffles, on large macropinosomes and in a Rab11-positive compartment of the endosomal pathway.

We also saw an increase in annexin 2-actin interactions as measured by FRET. In cells depleted of annexin 2 by siRNA, Src becomes phosphorylated but does not localise to the plasma membrane or to perinuclear vesicles. There was a reduction in phosphorylation of FAK on tyrosine 576, a key site associated with remodelling of focal adhesions, and the cells failed to transform. A phosphomimetic GFP-fusion of annexin 2 (Y23E-annexin 2-GFP) partially phenocopied cellular transformation by v-Src.

This is the first time that annexin 2 has been shown to be both an activator and an effector of v-Src. Disregulation of Src, annexin 2 and FAK have all been implicated in the progression of many forms of cancer, and of aggressive metastasis, a process highly dependent upon actin-remodelling, in particular.

This work places the proteins in the same pathway and ties together recent work from our and other laboratories which have implicated phosphorylation of annexin 2 in cytoskeletal remodelling (Rescher et al., 2008; Huber and Gulino-Debrac, 2008; de Graauw et al., 2008; Tatenhorst et al., 2006; Hayes et al., 2006).

de Graauw et al. (2008) Mol Cell. Biol. 28, 1029-1040.
Hayes et al. (2006) EMBO J. 25, 1816-1826.
Huber and Gulino-Debrac (2008) Mol. Cell. Biol. 28, 1657-1668.
Rescher et al. (2008) J. Cell Sci. 121, 2177-2185.
Tatenhorst et al. (2006) Neuropathol. Appl. Neurobiol. 32, 271-277.

**Keywords: annexin 2, Src kinases, tumor development, focal adhesion, actin**

# ANNEXIN A5, A PLURIPOTENT MEMBER OF THE ANNEXIN FAMILY OF $Ca^{2+}$-DEPENDENT PHOSPHOLIPID-BINDING PROTEINS

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Annexin A5 belongs to a multigene family of phospholipid binding proteins, the annexins. Annexin A5 was discovered as an anticoagulant protein with antithrombotic activity in vivo. Its anticoagulant activity arises from high-affinity binding to negatively charged phospholipids such as phosphatidylserine (PS) and subsequent multimerization on the lipid surface by homotypic interactions. To date, several other biological properties of annexin A5 have been described, including $Ca^{2+}$-channel activities, phospholipase A2 regulation, inhibition of phagocytosis of apoptotic cells by both activated and unactivated macrophages and immune modulation. In spite of available data, annexin A5's physiological function remains to be determined. It is generally accepted that its physiological significance is based on its abilities to bind PS and to crystallize on the phospholipid surface.

Due to its PS binding property annexin A5 has been developed succesfully into a Molecular Imaging agent to measure apoptosis in vitro and in vivo in animal models and in patients. The recent discovery that two-dimensional crystallization drives cellular uptake of annexin A5 has paved avenues to develop annexin A5 as a targeting vector in Targeted Drug Delivery strategies.

**Keywords: annexin A5, phospholipids, apoptosis**

# PLASMA MEMBRANE TARGETED ANNEXIN A6 AFFECTS STORE-OPERATED $Ca^{2+}$ ENTRY

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The annexins are a multigene family of $Ca^{2+}$- and charged phospholipid-binding proteins, which interact with the plasma membrane (PM) and the internal membrane systems upon increase of $[Ca^{2+}]_i$ or during intracellular acidification. However, the transient nature of the membrane binding of annexins complicates the study of their influence on intracellular pH and $Ca^{2+}$ homeostasis.

Therefore, we fused fluorescent protein-tagged annexins A1, A2 and A6 with H- and K-Ras plasma membrane targeting motifs to create proteins, which constitutively localise to the plasma membrane. Membrane anchors of the Ras isoforms direct the proteins to different microdomains within the plasma membrane: palmitoylated H-Ras tag (tH) to the lipid rafts, and polybasic K-Ras (tK) to non-raft regions. Constitutive PM targeting of annexin A6 with either of these sequences significantly attenuated the store-operated $Ca^{2+}$ entry (SOCE) in thapsigargin-treated cells. The effect was more pronounced in cells expressing annexin A6-tH than A6-tK. Notably, the PM targeting of annexin A6 with a H-Ras sequence also led to a reduction of the basal $[Ca^{2+}]_i$ and the diminished filling of the intracellular stores (ER).

This property was specific for annexin A6 as PM targeting of annexins A1 or A2 with tK and tH did not cause alterations in SOCE. For attenuation of SOCE, annexin A6 needed to be in close proximity to the plasma membrane, since the wild-type annexin A6, diffusely distributed in the cytoplasm, did not have any pronounced effect, indicating that the permanent PM localisation was necessary to amplify the local changes in $Ca^{2+}$ homeostasis. Removal of extracellular $Ca^{2+}$ further decreased the $[Ca^{2+}]_i$ in the annexin A6-tH cells, whereas $Ca^{2+}$ re-loading prior to stimulation restored basal $[Ca^{2+}]_i$ and store contents without affecting the SOCE reduction.

Our results implicate annexin A6 in regulation of SOCE, possibly by influencing the intracellular $Ca^{2+}$ extrusion mechanisms.

**Keywords: annexin A6, store-operated $Ca^{2+}$ entry**

# REGULATION OF $IP_3R1$ BY M-PHASE KINASES DURING OOCYTE MATURATION: OPTIMIZATION OF THE $Ca^{2+}$ RELEASE ACTIVITY AND OF THE ER ORGANIZATION

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To initiate embryo development, the sperm induces $Ca^{2+}$ oscillations in the egg that last for several hours. Those $Ca^{2+}$ oscillations result from the activity of the inositol 1,4,5-trisphosphate receptor ($IP_3R1$), the main $Ca^{2+}$-release channel localized in the endoplasmic reticulum (ER) of the mammalian oocyte. During oocyte maturation, an optimization occurs at the level of the $IP_3R1$ to allow the occurrence of a correct $Ca^{2+}$ signal at fertilization.

Our results indicate that $IP_3R1$ becomes phosphorylated during maturation at an MPM-2 epitope and that this persists until the fertilization-associated $Ca^{2+}$ oscillations cease. We also report that if maturation proceeds in the absence of ERK activity, $IP_3R1$ MPM-2 reactivity is diminished as well as $Ca^{2+}$ oscillations. However ERK is not directly phosphorylating the $IP_3R1$ at an MPM-2 epitope, as we demonstrate that Polo-like kinase 1 (Plk1), a conserved M-phase kinase, is the kinase phosphorylating $IP_3R1$ at an MPM-2 epitope. We also show that Plk1 and $IP_3R1$ interact in a M-phase preferential manner, and that they co-localize on the spindle poles and on other ooplasmic sites. The ERK pathway however regulates $IP_3R1$ cortical redistribution and the co-localization of $IP_3R1$ and Plk1 is reduced in the absence of ERK activity. The functional importance of Plk1-mediated phosphorylation of the $IP_3R1$ in the enhancement of its sensitivity during oocyte maturation and the phosphorylation consensus sites for Plk1 on the $IP_3R1$ are being investigated.

Besides phosphorylation, the $IP_3R1$ undergoes redistribution during oocyte maturation. We demonstrate that different branches of the cell cytoskeleton control this redistribution during oocyte maturation, and that the process is dependent on the meiotic stage. Moreover the $IP_3R1$ seems to play an unexpected but critical role in the reorganization of the ER during oocyte maturation, as down-regulation of $IP_3R1$ impedes the formation of ER cortical clusters in oocytes that have reached the MII stage.

Collectively, we propose that in mammalian oocytes the $IP_3R1$ plays a fundamental role both as a $Ca^{2+}$ channel and as an organizer of the ER.

**Keywords: oocyte maturation, fertilization, $IP_3$ receptor, MPM2 phosphorylation, Polo-like kinase 1, ERK**

# CALCIUM SIGNALLING IN EARLY DEVELOPMENT

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Calcium signals are well-known mediators of egg activation at fertilisation. In addition, calcium signals are found associated with cell division in some early embryos. In sea urchin embryos, cell cycle calcium signals are generated by inositol trisphosphate-mediated release of calcium from internal stores (Ciapa et al. 1994; Groigno & Whitaker 1998).

The major internal calcium store is the endoplasmic reticulum (ER): the spatial organization of the ER during mitosis may thus be important in shaping and defining calcium signals. In early Drosophila embryos, ER surrounds the nucleus and mitotic spindle during mitosis, rather than being dispersed throughout the mitotic spindle. This unusual ultrastructure offers an opportunity to determine whether perinuclear localization of ER conditions calcium signalling during mitosis.

We have established (Parry et al. 2005) that the nuclear divisions in syncytial Drosophila embryos are accompanied by both cortical and nuclear localized calcium transients. Constructs that chelate inositol trisphosphate also prevents nuclear division. Analysis of nuclear calcium concentrations demonstrates that nuclear calcium concentrations are differentially regulated. These observations demonstrate that mitotic calcium signals in Drosophila embryos are confined to mitotic microdomains and offer an explanation for the apparent absence of detectable global calcium signals during mitosis in some cell types.

Ciapa et al. (1994) Nature 368, 875-878.
Groigno & Whitaker (1998) Cell 92, 193-204.
Parry et al. (2005) J. Cell Biol. 171, 47-59.

**Keywords: mitosis, development, embryogenesis, Drosophila, $Ca^{2+}$ microdomain, $Ca^{2+}$ signalling**

# HOW DOES CALCIUM REGULATE CARDIAC HYPERTROPHY?

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In non-contractile cell types increased intracellular $Ca^{2+}$ concentration directly regulates proliferation, differentiation, metabolism, motility, and cell death through the action of unique $Ca^{2+}$-dependent signaling proteins. Many of these $Ca^{2+}$-dependent regulatory processes are conserved in cardiac myocytes although the physiologic relevance of "reactive" $Ca^{2+}$ -signaling remains a nebulous concept in the heart given the highly specialized manner in which $Ca^{2+}$ cycling is regulated for excitation contraction-coupling (ECC).

While it is theoretically possible that total contractile $Ca^{2+}$ associated with ECC regulates $Ca^{2+}$-sensitive signaling proteins such as calcineurin, CaMK and PKC$\alpha/\beta/\gamma$, it seems unlikely since such a mechanism would not discriminate between inotropy and signaling. Indeed, near maximal $Ca^{2+}$ cycling due to phospholamban deletion in mice does not induce reactive signaling pathways or hypertrophy, indicating that inotropic drive may not be a direct source of $Ca^{2+}$ for intracellular signaling pathways. Thus, specialized pools of $Ca^{2+}$ that are location-specific or somehow buffered from cytoplasmic $Ca^{2+}$ need to be evoked to account for the regulation of $Ca^{2+}$-sensitive signaling proteins such as calcineurin.

To address this issue we have generated transgenic and gene-targeted mice with altered L-type and T-type $Ca^{2+}$ channel activity in the heart, as well as mice with a specific reduction in sub-sarcolemmal $Ca^{2+}$, and alterations in TRPC channels. These various animal models suggest that calcineurin signaling and reactive hypertrophy are regulated by a distinct microdomain of $Ca^{2+}$ in the sub-sarcolemmal region of a cardiac myocyte that is distinct from contractile $Ca^{2+}$ associated with ECC. We determined that increased L-type $Ca^{2+}$ channel activity and TRPC channel activity are pathologic where it induces cellular necrosis and hypertrophy, yet paradoxically, increased T-type $Ca^{2+}$ channel activity appears to be protective where it antagonizes hypertrophic signaling.

Thus, channel-specific, non-junctional microdomains of $Ca^{2+}$ appear to regulate distinct signaling pathways involved in cardiac adaptation or maladaptation.

**Keywords: cardiac hypertrophy, $Ca^{2+}$ microdomain, calcineurin, L-type $Ca^{2+}$ channels, T-type $Ca^{2+}$ channels**

# RNA INTERFERENCE TARGETING STIM1 SUPPRESSES VASCULAR SMOOTH MUSCLE CELL PROLIFERATION AND NEOINTIMA FORMATION IN THE RAT

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Our objective was to study the expression and function of stromal interaction molecule 1 (STIM1), an endoplasmic reticulum protein recently identified as the calcium sensor that regulated $Ca^{2+}$ release-activated channels in T cells.

Methods and results: STIM1 was up-regulated in serum-induced proliferating human coronary smooth muscle cells (hCASMC) as well as in the neo-intima of injured rat carotid arteries. Growth factors-induced proliferation was significantly lower in hCASMC transfected with STIM1 siRNA than in those transfected with scrambled siRNA (increase relative to 0.1% S: 116±12% and 184±16% respectively, $p<0.01$). To assess the role of STIM1 in preventing vascular smooth muscle cells (VSMC) proliferation in vivo, we infected balloon-injured rat carotid arteries with an adenoviral vector expressing a short hairpin (sh) RNA against rat STIM1 mRNA (Ad-shSTIM1). Intima/Media ratios reflecting the degree of restenosis were significantly lower in Ad-shSTIM1-infected arteries than in Ad-shLuciferase-infected arteries (0.50±0.04 vs 1.06±0.17, $p<0.0005$). Finally, we demonstrated that silencing STIM1 prevents activation of the transcription factor NFAT and activates CREB, the cAMP-responsive element binding protein.

In conclusion, STIM1 appears as a major regulator of in vitro and in vivo VSMC proliferation, representing a novel and original pharmacological target for prominent vascular proliferative diseases.

**Keywords: STIM1, TRPC, vascular myocytes**

# TRPC1 CHANNEL IS REQUIRED FOR CHEMOTAXIS TOWARDS FGF-2

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Chemotaxis describes the ability of cells to migrate along the concentration gradient of chemical attractants. It plays a critical role in immune cell response and tumor metastasis. The process of migration itself can be modeled as a repeated cycle of protrusion of the cell front and subsequent retraction of the rear part, the intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) being one of the major regulators for coordinating the movement of these two cell poles with each other. $[Ca^{2+}]_i$ of migrating cells strongly depends on $Ca^{2+}$ influx through $Ca^{2+}$ channels some of which were suggested to be mechanosensitive. The molecular identity of these channels, however, is largely unknown.

Here we tested whether the putative mechanosensitive TRPC1 channel could be involved in coordinating different components of the cellular migration machinery and thereby be required for directed migration. To this end, we used transformed renal epithelial MDCK-F-cells with genetically altered TRPC1 expression. Reducing TRPC1 expression leads to a strong impairment of persistent migration since the cells protrude multiple lamellipodia simultaneously into opposing directions. In most of these cells, the position of the microtubule organizing centre with respect to the nucleus does not indicate the direction of migration. When exposed to a chemical gradient of the chemokine fibroblast growth factor 2 (FGF-2), cells with reduced TRPC1 expression are not able to migrate into the direction of higher FGF-2 concentrations, whereas control cells and TRPC1-overexpressing cells are able to do so. The effect of TRPC1 knockdown on chemotaxis is mimicked by the application of the spider venom GsMTx-4, an inhibitor of mechanosensitive cation channels. These findings indicate that TRPC1 channels play an important role in coordinating the dynamic behavior of front and rear parts of migrating cells thereby making directed migration / chemotaxis possible.

**Keywords: TRPC1, chemotaxis, migration**

# CALCIUM AND CANCER: TARGETING $Ca^{2+}$ TRANSPORT

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Prostate cancer is associated with the appearance of new cell phenotypes, characterized by apoptosis inhibition and aberrant cell proliferation. Deregulated cell differentiation and proliferation together with the suppression of apoptosis provide the condition for abnormal tissue growth, which ultimately can turn into uncontrolled expansion and invasion characteristic of cancer.

The role of $Ca^{2+}$ in the overall of cancer-related cell signalling pathways is uncontested. Alterations in $Ca^{2+}$ homeostasis have been described to increase proliferation, to induce differentiation or apoptosis.

The aim of our present work was to establish the role and regulation of $Ca^{2+}$-permeable ion channels in prostate carcinogenesis.

1. We have demonstrated that a $Ca^{2+}$ signal can promote either cell proliferation or apoptosis, depending on the type of channel involved: $Ca^{2+}$ entry via TRPC6 and TRPV6 channels stimulate cell proliferation whereas ORAI1 and TRPC1 are mostly involved in apoptosis induction.

2. We were particularly interested by TRPM8 channels since TRPM8 is a target gene of the androgen receptor and its expression strongly increases in prostate cancer. Recent evidence we have obtained indicate that TRPM8 may be expressed not just in the plasma membrane, but also in the endoplasmic reticulum (ER) membrane where TRPM8 may operate as an ER $Ca^{2+}$ release channel. We propose a model for the differential regulation and functional significance of plasma membrane and ER TRPM8 in carcinogenesis.

Recent progress achieved in our understanding of molecular mechanisms of TRP channels signalling involved in the control of prostate cancer progression ensures that sooner or later fundamental breakthroughs will make their way to practical implications.

**Keywords: proliferation, differentiation, TRPC channels, TRPM8**

# CALCIUM AND CELL DEATH

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$Ca^{2+}$ signals are important mediators of apoptosis and are regulated by both anti-apoptotic and pro-apoptotic members of the Bcl-2 protein family.

Our work has focused on the regulation of $IP_3$-mediated $Ca^{2+}$ signals by the anti-apoptotic protein Bcl-2 in T cells (Chen et al., 2004; Zhong et al., 2006). $IP_3$-mediated $Ca^{2+}$ signals are generated in response to T cell receptor (TCR) activation and the pattern of these $Ca^{2+}$ signals varies according to the strength of TCR activation (Zhong et al., 2006). Strong TCR activation induces sustained $Ca^{2+}$ elevation that triggers apoptosis, whereas weak TCR activation generates $Ca^{2+}$ oscillations that favor cell survival. Bcl-2 differentially regulates these $Ca^{2+}$ signals, inhibiting sustained $Ca^{2+}$ elevation but enhancing $Ca^{2+}$ oscillations.

The inhibition of sustained $Ca^{2+}$ elevation by Bcl-2, and hence the inhibition of TCR-induced apoptosis by Bcl-2, is mediated through a direct physical interaction of Bcl-2 with the $IP_3$ receptor ($IP_3R$) (Chen et al., 2004; Rong et al., 2008). This interaction is detected in cell extracts by Bcl-2-$IP_3R$ co-immunoprecipitation and by binding of natural and recombinant Bcl-2 to GST-$IP_3R$ fragments. The interaction is also detected within intact cells by FRET. The site of Bcl-2 interaction on the $IP_3R$ maps to an 80 amino acids region within the regulatory and coupling domain (Rong et al., 2008). A 20 amino acid synthetic peptide corresponding to a sequence within this $IP_3R$ region displaces Bcl-2 from the $IP_3R$ and reverses Bcl-2's inhibition of $IP_3R$ channel opening in vitro. When introduced into Bcl-2 positive T cells, this peptide reverses Bcl-2's inhibition of $IP_3$-mediated $Ca^{2+}$ release from the endoplasmic reticulum and enhances both TCR-induced $Ca^{2+}$ elevation and apoptosis.

Overall, these findings indicate the importance of the Bcl-2-IP3R interaction in modulating $IP_3$-mediated $Ca^{2+}$ signals and apoptosis. They also suggest the potential of targeting the Bcl-2-$IP_3R$ interaction for therapeutic purposes in diseases such as cancer where Bcl-2 overexpression inhibits apoptosis.

Chen et al. (2004) J. Cell Biol. 166, 193-203.
Rong et al. (2008) Mol. Cell 31, 255-265.
Zhong et al. (2006) J. Cell Biol. 172, 127-137.

**Keywords: Bcl-2, $IP_3R$, apoptosis**

# INOSITOL TRISPHOSPHATE AND CALCIUM SIGNALLING

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The discovery that inositol trisphosphate ($IP_3$) was a $Ca^{2+}$-mobilizing second messenger emerged from a series of experiments on the blowfly salivary gland. Electrophysiological experiments had revealed that an internal supply of $Ca^{2+}$ was essential for controlling the chloride flux necessary to maintain fluid secretion. An investigation into the role of the inositol lipid signalling pathway led to the suggestion that $IP_3$, which was one of the products of $PIP_2$ hydrolysis, might function as a second messenger to release $Ca^{2+}$ from an internal store (Berridge, 1983). This suggestion was then confirmed by showing that $IP_3$ would mobilize $Ca^{2+}$ from permeabilized pancreatic acinar cells (Streb et al., 1983).

Subsequent studies have confirmed that throughout the life cycle of a typical cell many different cellular processes are controlled by this $IP_3/Ca^{2+}$ signalling system. Its first role is to mediate the process of fertilization and it then continues to function throughout development to control events such as axis specification. Once the organism has formed, $IP_3/Ca^{2+}$ continues its control function by regulating the activity of differentiated cells such as smooth muscle contraction, modulation of cardiac cell contractility, proliferation of lymphocytes, secretion by exocrine glands and the modulation of neural activity during learning and memory. Finally, $IP_3$ may contribute to the control of cell death by apoptosis by regulating the flux of $Ca^{2+}$ between the endoplasmic reticulum and mitochondria. To carry out so many control functions, the $Ca^{2+}$ system needs to be highly versatile (Berridge et al., 2000). This lecture will explore how much of this versatility is achieved through the operation of this $IP_3/Ca^{2+}$ signalling system.

Berridge (1983) Biochem. J. 212, 849-858.
Berridge et al. (2000) Nature Rev. Mol. Cell Biol. 1, 11-21.
Streb et al. (1983) Nature (Lond.) 306, 67-69.

**Keywords: $IP_3$, $Ca^{2+}$ signalling**