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Meeting Report

The 9th Meeting of the European Calcium Society

Strasbourg, France

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INTRODUCTION

Strasbourg—bounded by the Ill River, canals cascading through the old quarter of La Petite France, former residence of Johann Gutenberg, Louis Pasteur and Albert Schweitzer and now home to the Council of Europe, European Parliament and the European Court of Human Rights, and this summer's host of the *9th Meeting of the European Calcium Society* (ECS). Congratulations to the meeting organizers for another outstanding scientific venue held in the vibrant setting of Strasbourg and the Campus Illkirch.

Considering its origins as a meeting devoted primarily to the topic of calcium-binding proteins, and a modest number at that, the breadth of the 9th meeting of the ECS was remarkable. Calcium channels, pumps, a variety of EF-hand calcium-binding proteins, annexins, human pathologies, therapeutics—from plants and insects to animals and humans. This short review will try to capture some of the diversity represented at this year's meeting.

THE EVOLUTION OF CALCIUM SIGNALING

Bob Williams (UK) thinks big—from clouds to brains—his presentation this year about the unique role of calcium ions in evolution was meant to challenge the audience, periodically tweaking the collective noses of molecular biologists, and provide a context for the remaining presentations which focused on very specific aspects of calcium signaling.

Bob reminded us that early cells excluded such elements as sodium, chloride and manganese, while retaining others like potassium and magnesium. Calcium, owing to its ability to complex with other elements resulting in the formation of precipitates, had to be excluded from the cytoplasm, where concentrations greater than 10^{-5} M are toxic. Perhaps the earliest calcium-related proteins were involved in restricting calcium entrance into the cell and transferring leaked calcium back out of the cell.

Calcium has unique coordination properties compared to other physiological metal ions. These properties include its size, electrostatic nature for selectivity and fast on and off rates for regulation. In addition, calcium ions are able to switch oxidation states, working closely with Mn^{2+}, as seen in photosystems. The gradual oxidation of the environment, while disastrous for the early anaerobic procaryotes, provided a milieu advantageous to the evolution of eucaryotes, comprised of different compartments capable of conducting chemistries previously omitted from procaryotes. While serving primarily a protective function in prokaryotic cells, calcium became essential for temporal and spatial signaling underlying communication between different cellular compartments and the extracellular environment in eukaryotic systems.

Calcium-dependent communication allowed for fast signaling relatively independent of the host's genome. Having been excluded from the cell originally, calcium gradients could now be put to work. The variety of proteins responsive to calcium provided more options in signaling, thus contributing to the evolution of more complex cells and systems.

The one hour keynote lecture by Michael Berridge (UK) elaborated upon the versatility of calcium signaling and some of the underlying signaling components. Sir Michael reminded us that calcium can operate in microseconds to drive exocytosis in synaptic terminals, in milliseconds for muscular contraction, in seconds or minutes for adjustments to metabolism and in hours for fertilization. Evolution and the evolution of calcium signaling are thus intimately intertwined.

Sir Michael emphasized that through an enormous calcium signaling toolkit made of various on and off systems, each cell through differentiation selects its own signalsome. He elegantly illustrated this concept by comparing the diverse molecular requirements for calcium signaling during contraction in cardiac cells versus the requirements during the

activation of T cells or the binding of phytohemaglutinins in plant cells. The message was that each cell has its own calcium signaling signature. The next part of Sir Michael's presentation dealt with temporal aspects of calcium signaling, and again a comparison between diverse cells provided clear evidence of the operational differences between, for example, simple calcium transients that arise upon stimulation of the cell and those of a permanent calcium oscillating system, wherein more complex information is encoded by the frequency, amplitude and shape of the signaling. As far as spatial aspects, Sir Michael pointed to the advancements in calcium imaging and in particular to the improved sensitivity of calcium measurements. These advances have allowed calcium transients to be visualized at single channels, revealing an array of signals described as sparks, puffs, elementary events linked to AMPA receptors, sparklets, syntilla and blinks. The significance of such discrete microdomains of calcium transients was illustrated when Sir Michael showed how ventricular cells rely on elementary events, while other cells (atrial cells, for example) build blocks of elementary events to produce more global signals. In the last part of his talk, Sir Michael examined how various diseases may arise through changes in calcium signaling. The basic idea being that cell specific signalsomes can be modified through genotypic and phenotypic remodeling. For genotypic remodeling there are a growing number of diseases in which genes involved in calcium signaling are impaired. The consequences of such mutations are easy to appreciate. The concept of phenotypic stability and remodeling is more subtle. Diseases such as manic depression and cardiac hypertrophy may arise through changes in the levels of expression of different signalosome components. Manic depression may occur when the signalosome controlling the IP3 system within neurons is altered, leading to excessive metabotropic receptor signaling and greater hyperactivity. The idea is supported by looking at the mechanisms of lithium and valproate, two drugs used to stabilize the disease by acting on enzymes which decrease cerebellar inositol and therefore act to remodel IP3 homeostasis.

The involvement of calcium and components of its signaling pathways in human pathologies then developed into a central theme of the meeting.

DIVERSE CALCIUM PATHWAYS LEADING TO HUMAN DISEASE

The diagnosis of melanoma is often substantiated by the immunohistochemical staining of S100B, however, the significance of elevated S100B has gone largely unappreciated. David Weber (USA) postulated a mechanism for S100B action that may have ramifications beyond skin cancer. Weber described the calcium-dependent binding of S100B to wild type p53, disrupting p53 oligomers, and thus promoting p53 degradation and interfering with its function as a tumor suppressor. Weber and his colleagues show that the downregulation of S100B by means of siRNA technology can restore p53 protein levels and concomitantly its apoptotic properties. Based on this finding, peptides and other small molecules can be devised to interfere with the binding of S100B, thus allowing p53 to function in the apoptotic activation of cancer cells. By combining structural information and computer simulations, screening a small molecule database aided in the identification of potential inhibitors of S100B-p53 interaction. In vitro binding assays were followed by measurements of the ability of each inhibitor to interfere with the growth of melanoma cells. Further structural analysis then led to the synthesis of drug derivatives even better suited as inhibitors. This approach identified a series of small molecules, including pentamidine, that enter cells, bind S100B, block p53 binding, restore p53 and p21 activity, and decrease melanoma growth in mice. The site of interaction between S100B and p53 thus is proving to be a viable target for the treatment of some cancers.

The discussion of S100 proteins and their involvement in human disease continued with the presentation by Arnaud Galichet (Switzerland). Several S100 proteins, including S100A6 and S100B, are expressed at aberrant levels in different human maladies, including Alzheimer's disease. In grey matter S100A6 is associated with astrocytes surrounding amyloid deposits of senile plaques. Since S100A6 has high affinity for zinc, its over-expression may underlie the deposition of zinc in these pathological bodies. Galichet and colleagues now report that in a model using SH-SY5Y human neuroblastoma cells, addition of S100B enhances proliferation and cell survival. Conversely, S100A6 decreases cell viability by activating pro-apoptotic pathways. Looking for a mechanism of action, Galichet described the interaction of S100 proteins with RAGE, the Receptor for Advanced Glycation End products. Blocking RAGE activity using the soluble form of RAGE or using specific RAGE antibodies reverses the effects of S100B and S100A6, indicating that these proteins may regulate the activity of RAGE and subsequently the proliferation and survival of cells. The site of interaction between RAGE and S100 proteins was characterized further by Michael Koch and his colleagues (Germany). The group first expressed the different Ig domains of RAGE (V, C1 and C2), and then demonstrated that S100B binding to RAGE is calcium and zinc dependent, and the site of interaction is specifically at the RAGE V-domain. Binding of S100B, perhaps in a tetrameric if not dimeric form, instigates the oligomerization of RAGE, a necessary step in the receptor's activation. X-ray diffraction studies of the extracellular domain of RAGE are underway and should reveal more about the binding and activation mechanisms of S100B, elaborating upon additional sites for therapeutic intervention based on S100 protein binding.

The role of S100 proteins in human disease was not confined to oral presentations. In the poster session Berit Hoj (Denmark) presented data that ALG-2, originally identified as a pro-apoptotic factor, may have the opposite effect in some cancer cells where it is over-expressed. The investigators manipulated ALG-2 levels in U2OS cells using different siRNA molecules. The downregulation of ALG-2 reduced proliferation. It also enhanced cell sensitivity to drugs like thapsigargin, thereby diminishing cell viability. ALG-2 appears to act like a thermostat assessing calcium homeostasis and then impacting on the decision of the cell to persist or die. The decision relies on the levels of calcium and ALG-2, and the pathways specific to each cell in which ALG-2 participates. As demonstrated by Hoj and colleagues, ALG-2 levels and its pathways may vary between cell types.

Calcium-related diseases go beyond the S100 proteins. Philippe Lory (France) described a growing array of calcium channelopathies, which now includes neuromuscular diseases such as Lambert-Eaton myasthenia gravis and blinding diseases including forms of congenital stationary night blindness. While mutations can produce changes in the electrophysiological properties of the individual calcium channels in some cases, mutations also cause changes in protein synthesis, modification, trafficking and stability, and thereby have demonstrable effects on channel function. Lory described truncated forms of Cav2.1 that exert a dominant negative effect in episodic ataxia, a cerebellar dysfunction leading to intermittent upper body impairment. Expression of the truncated form of this P-Q type of high voltage channel in transient transfection experiments resulted in loss of detectable calcium current in spite of the expression of wild-type

channels. Lory and colleagues were able to determine that neither the mutant or wild-type channels reached the surface of the cell. Total channel protein is reduced, likely as a result of misfolding of the mutant protein in the endoplasmic reticulum, its association with wild-type channel and early degradation of the pair. Using a similar experimental approach, these investigators also demonstrated that mutant forms of the T-type channel Cav3.2, found in childhood absence epilepsy, actually increase their density in the plasma membrane. Such mutations do not increase overall channel expression, rather the targeting of the channel and its density at the surface of the cell increase, resulting in a gain of function. Channelopathies such as these demonstrate that too much or too little of a specific calcium channel can be deleterious. By examining such mutations, investigators are gaining insight into the functional domains of various channels, as well as the underlying etiologies of disparate human diseases. The two may lead to novel therapies.

From calcium-binding proteins to channels to calcium itself—while responsible for the contraction of myocytes during each beat of the heart, calcium also can instigate hypertrophy, the increase in muscle mass of the heart from cell enlargement. While adaptive in the case of exercise, hypertrophy is a leading contributor to human cardiovascular disease and death. H. Llewelyn Roderick (UK) described the role of InsP3 in the release of calcium from perinuclear regions in myocytes; these events are distinct from the bulk calcium changes associated with contraction. InsP3-induced calcium changes contribute to hypertrophy by activating L-type voltage-operated channels and by β-adrenergic stimulation. Roderick and colleagues demonstrated that InsP3-induced calcium release activates the expression of hypertrophy-related genes that can contribute to the increases in myocyte size. One such gene regulated by InsP3-induced calcium release is ANF, the atrial natriuretic factor, a polypeptide hormone that acts to reduce the water, sodium and adipose loads on the circulatory system, thereby returning blood pressure to normal levels. Blocking InsP3-induced calcium release appears to interfere with the expression of ANF, likely making myocytes more susceptible to hypertrophy.

The understanding of various calcium pathways can lead to innovative therapies. As a further example, Andreas Guse (Germany) reported on the formation of nicotinic acid adenine dinucleotide (NAADP) upon activation of the T cell receptor/CD3 complex. NAADP is a potent activator of calcium release from within the cell, but receptor-mediated generation of NAADP had not been previously demonstrated. Guse reported that NAADP stimulated the mobilization of calcium in a ryanodine receptor-dependent manner, supported by studies of T cell clones lacking receptors. The group then synthesized a host of NAADP antagonists which could block calcium signaling and the subsequent activation of T cells in vitro. These antagonists demonstrated a therapeutic effect in a rodent model of multiple sclerosis.

Continuing with the theme of therapeutics, D. Martin Watterson (USA) provided a sobering review of the status of new drug development. Despite considerable funding for drug discovery, there has been a decline in successful research bridging basic science studies with the implementation of new therapeutics. In part the problem arises from an emphasis on in vitro studies that do not mimic in vivo conditions. In addition the problem has increased with the development of high throughput screening aimed at single targets. The specificity of drugs determined using isolated targets ignores the complexity of multifactorial diseases, so single target-based drug development often leads to ineffective therapies. The alternative is to develop strategies from studies of known drugs that meet the needs for bioavailability, safety and effectiveness and couple that information with earlier stages of in vivo testing. Finally, the remodeling of candidate drugs can then be optimized taking advantage of high throughput technologies. A proof-of-principle of the approach was demonstrated initially for the development of MW01-5-188WH, a unique anti-inflammatory compound that is orally bioavailable and brain penetrant and protective against pathologies associated with Alzheimer's disease. Watterson and colleagues are now targeting the calcium and calmodulin-dependent kinases, MLCK and DAPK, more challenging targets owing in part to their substrate requirements and therefore more structural limitations for the design of inhibitors.

While we try to synthesize new compounds, we can't overlook millions of years of evolution. Resveratrol, a natural plant product, has been implicated in cardioprotection, cancer prevention and to some extent cancer treatment. Jacobo Elies (Spain) presented data in vascular smooth muscle cells that resveratrol, both cis and trans isomers, causes an increase in intracellular calcium. Pharmacologic studies indicate that resveratrol releases calcium from thapsigargin-sensitive stores. Resveratrol is non-toxic at levels likely to be therapeutic. Also, remodeling of the compound could enhance bioavailability. Alternatively, new modes of drug delivery could be paramount to effective treatment. If so, resveratrol could activate a number of the pathways being studied by many of the presenters at this year's ECS meeting, instigating cancer cell death or protecting neurons during degenerative events. A major source of resveratrol is red wine—something we learn to appreciate even more at each of the ECS meetings.

CALCIUM CHANNELS AND TRAFFICKING

Once again, the ECS meeting captured the current hot topics and major developments in calcium signaling. Don Gill (USA) and Jim Putney (USA) added new links to the pathway underlying the activation of capacitative calcium entry through store-operated calcium channels (SOCs). STIM (the Stromal Interaction Molecule), was initially identified using an RNAi-based screen of a subset of *Drosophila* genes to find components of the pathway linking intracellular calcium stores to the capacitative entry of calcium through the plasma membrane. STIM encodes a single transmembrane-spanning calcium-binding protein. STIM1, the human homologue, was reported to be an essential component of SOC and the calcium-release activated calcium (CRAC) channel. STIM1 functions as a calcium sensor that activates the CRAC channel. Expression of STIM1 EF-hand mutants incapable of coordinating calcium results in constitutive activation of the CRAC channel.

Further genome-wide RNAi screens in *Drosophila* and mammalian cells by Feske et al. and Vig et al. led to the identification of Oria1 or CRAM. The essential role of Orai1 was demonstrated following its RNAi suppression resulting in a significant reduction in store-operated calcium entry. The Gill group reported that in addition to being a sensor within calcium stores, STIM1 may also function within the plasma membrane, perhaps controlling the operation of calcium entry itself. Co-expression of STIM1 with Orai1 was reported to recapitulate the CRAC current.

Interestingly, over-expression of Orai1 or STIM 2 suppresses SOC activity. The Putney group reported that the two other mammalian Orai1 homologues, Orai2 and Orai3, were able to augment store-operated calcium entry but to a lesser extent than Orai1. Instead of

incorporating into the surface membrane, STIM1 was reported to interact or regulate Orai1 at sites of close apposition between the plasma membrane and the ER-like compartment in which STIM1 resides. Recent publications continue to define both the role of STIM1 in the activation of native SOC and TRPC1 channels and the role of Orai1 as a pore subunit of the CRAC channel essential for ion selectivity. No doubt, the discovery and characterization of STIM1 and associated molecules have produced a strong pulse in the calcium signaling field. It also clearly extends the already diverse continuum of EF-hand proteins. This time, a single EF-hand motif, instead of tightly coupled EF-hand motifs, plays an essential role in calcium signaling.

The role of calcium in various intracellular compartments such as the ER, Golgi apparatus and membranous organelles of the secretory pathway was described by Frank Wuytak (Belgium). The P-type SPCA (secretory pathway calcium ATPase) pumps were shown to be evolutionarily conserved. SPCA1 is expressed in most mammalian cells, and mutation of different spliced SPCA1 variants is associated with Hailey-Hailey disease, a blistering skin disorder in humans owing in part to insufficient calcium needed for the proper assembly of desmosomes between neighboring skin cells. Severe malformations and loss of pigmentation also are observed following the knockdown of SPCA in zebrafish embryos, suggesting an essential role of SPCA in embryonic development as well.

According to Wuytak, due to their higher affinity for calcium and lower catalytic turnover rate compared to SERCA, SPCA1 and SPCA 2 are essential for calcium and manganese transport/detoxification. Wuytak and associates also reported the effect of saponin permeabilization on the morphology of the ER and Golgi of COS-1 cells. We're sure to learn more about the topic, as the next calcium binding meeting will be organized by Wuytack and held in Leuven, Belgium.

CALCIUM SIGNALING IN GENE EXPRESSION

Following the theme of diversity, M.R. Kreutz (Germany) discussed the role of caldendrin in controlling the NMDA receptor-activated morphogenetic signaling to the nucleus. Caldendrin (also called CBP-1), with a strong structural similarity to calmodulin, is a postsynaptic density component, and its translocation to the synapse depends on activity. Caldendrin and its binding partner Jacob are subject to a calcium-dependent co-localization in a subset of spine synapses. The observation of a rapid change of morphology of the dendritic tree and synaptic contacts upon NMDA stimulation was attributed to the relocation of Jacob to the nucleus by importinα." Instead of calmodulin, caldendrin regulates this translocation process by competitive binding of the NLS region of Jacob, which in turn blocks the interaction with Importinα." Therefore, the protein complex of Jacob-caldendrin functions as a liaison in controlling NMDA receptor-activated synapse to nucleus communication and synapto-dendritic cytoarchitecture.

M. Savingnac (France) reported the possible role of DREAM (Downstream Regulatory Element Antagonist Modulator) in the function of B lymphocytes. The mutation of EF-hand calcium-binding motifs within DREAM results in this transcriptional repressor becoming insensitive to the change of nuclear calcium, thus leaving it complexed with DNA during calcium stimulation. T-cells isolated from transgenic mice expressing the dominant active mutant of DREAM displayed lower levels of cytokine production following activation. DREAM was shown to remain bound to these cytokine promoter regions repressing transcription. DREAM may thus act as a calcium-dependent repressor in T-cells. The group is now extending their studies to the role of DREAM during immunoglobulin production in B-cells.

In a short talk and several posters, Rosario Donato (Italy) and his colleagues presented rapid progress in unraveling the diverse biological functions of the calcium-modulated EF-hand protein S100 B. Comparing the stable over-expression of S100B with data obtained through RNA interference, his group demonstrated that intracellularly S100B is able to stimulate proliferation and inhibit differentiation of non-nervous cell myoblasts by inhibiting p38 MARK. By interfering with MyoD expression in a NF-κB-dependent manner, S100B regulates myotube formation, possibly playing a dual role during embryonic myogenesis and muscle regeneration. In addition, extracellular S100B was shown to bind to RAGE, stimulate COX-2 expression and TNF-β release during BV-2 microglia activation. To further explore the mechanism of activation, they demonstrated that inhibition of S100 B expression reduces proliferation and stellation in other glial types.

Following this year's theme of diversity, one of the first ECS presentations on Drosophila was given by Robert Schulz (USA). Following Dr. Klee's review of the calcineurin story, Schulz showed that mutations in one of the calcineurin B2 genes in fruit flies result in the collapse of indirect flight muscles, akin to the phenotype seen with mutations in Tn1, and a decrease in the expression of the myosin heavy chain (mhc) gene. Functional and regulatory interactions were shown between genes encoding canB2 and mhc protein. Mutants of Drosophila defective in the canB2 gene demonstrate hypercontractability of the indirect flight muscle, ascribed to dysfunction of the interaction between thick and thin filaments, and lower expression levels of the mhc protein. Schulz and colleagues are now trying to identify a calcineurin-sensitive transcriptional enhancer for the mhc gene in order to further characterize the calcineurin-sensitive governance of mhc expression.

A PROPER FINISH

Meeting participants were treated by Dr. Claude Klee (USA) to an informative review of calcium-regulated proteins and especially calcineurin. Dr. Klee reminded the audience of how critical data can go unappreciated for decades. Following Ringer's original experiments in the 1880s demonstrating the importance of calcium during contraction of the heart, little work was done in the area until the mid-1960s when troponin C was isolated and the exquisite molecular model of muscle contraction developed. In the same era it became evident that calcium, in addition to muscle contraction, also controlled cyclic nucleotide phosphodiesterases, and that the reaction was regulated by a factor, later identified as calmodulin. The field exploded with the recognition that a multitude of proteins, including kinases, cyclases and cytoskeletal elements were regulated by calcium and calmodulin. Once parvalbumin was crystallized, the structural basis of calcium binding, the EF-hand, was discerned, and it became clear how the motif contributed to calcium binding in troponin C, calmodulin and the ever-expanding family of calcium-binding proteins. An appreciation developed for the complexity of multiple calcium binding sites in such proteins as calmodulin, and how the affinities of such sites varied and could alter upon binding to target. Our meeting host, Dr. Jacques Haiech, was a prominent figure in these studies.

Of the many proteins that could be eluted from a calmodulin column, Dr. Klee and her colleagues focused on one, identified as calcineurin. Although the first calcium- and calmodulin-dependent phosphatase, the significance of calcineurin was realized much later when it became apparent that it was the target of the immunosuppressant drugs FK506 and then cyclosporin A. Years of initial investigation of calcineurin provided structural data, characterization of the interaction between its catalytic and regulatory subunits, the roles of calcium and calmodulin, and the description of its rather ubiquitous expression from yeast to humans. Function, however, was elusive until immunosuppressant drugs were shown to be specific inhibitors of calcineurin. This led to studies of calcineurin-dependent protein dephosphorylation and the identification of NFAT as a calcineurin substrate in T cells. Dephosphorylation by calcineurin activates NFAT's translocation from the cytoplasm to the nucleus where it interacts with AP1 and leads to the expression of interleukin during the early stage of T-cell activation. This was one of the first signal transduction pathways to be elucidated. In addition to its role in T-cell activation, calcineurin is now implicated in a variety of neurological, immunological and cardiovascular disorders. All of this and more by following one protein from a calmodulin column—history remains an excellent teacher—thank you Dr. Klee.

2008 in Leuven—plan on being a part of the continuing excitement!