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Meeting Report

Calcium signal transduction and cellular control mechanisms

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1. Introduction

The 8th Meeting of the European Calcium Society, held at Hinxton Hall near Cambridge from 28–31 July 2004, provided a unique snapshot of the current status of the many and varied aspects of Ca^{2+} signalling. The scientific program reflected this large and diverse field, it dealt with the generation, detection and the function of Ca^{2+} signals in a wide range of cellular processes including exocytosis, contraction, cell proliferation, development and apoptosis. Throughout the meeting there were numerous reminders of how dysregulation of Ca^{2+} signalling can result in disease.

2. Generation of Ca^{2+} signals

An increase in the intracellular level of Ca^{2+} depends upon entry channels in the plasma membrane and release channels in the membranes of the internal stores, the endoplasmic reticulum (ER) or the sarcoplasmic reticulum (SR). **Don Gill**, who gave the plenary lecture, dealt with the entry of external Ca^{2+} through store-operated channels (SOCs) and transient receptor protein (TRP) channels. An important point to emerge from the lecture was the need to use more physiologically relevant stimuli when considering these entry channels particularly the SOCs. The classical method of using the SERCA pump inhibitor thapsigargin to deplete the ER pool is questionable, as it may also induce a cell stress response. Just how an empty store activates entry remains an unsolved problem but Gill described recent experiments that implicate the inositol 1,4,5-trisphosphate receptor ($InsP_3R$) in the activation of the canonical TRPC3 isoform channels, suggesting a possible relationship between the SOCs and TRPs. **Jim Putney** also dealt with how certain isoforms of the canonical TRPC channels are activated. He described how stimulation of muscarinic receptors activated TRPC3 channels expressed in HEK293 cells using diacylglycerol (DAG) as a second messenger. While this seems to be a clear-cut experiment, Putney raised the spectre of expression artefacts. For example, he described how TRPC3 channels act like SOCs when expressed at low levels in DT40 cells but were transformed into DAG-sensitive channels at high expression levels. Similar ambiguous results were obtained when entry was studied in cells using either transient or stable transfections. While we have learnt a lot about the properties of the TRP channels we still have much to learn about exactly how they are activated and whether or not any of them function as SOC channels.

Daniela Pietrobon also drew attention to the problems of using expression systems to characterize Ca^{2+} entry through voltage operated Ca^{2+} channels (VOCs). She was interested in studying the properties of the mutant Ca(v)2.1 channels (P/Q type channels), which are responsible for familial hemiplegic migraine type 1 (FHM-1). By studying the properties of these channels in their natural environment (i.e. the cerebellar neurons), she was able to show that the channels open more readily and at lower voltages resulting in much larger current densities. This shift in the activation properties results in an increase in the excitability of the cortical neurons that could explain the onset of migraine.

The main channels responsible for releasing Ca^{2+} from internal stores are the ryanodine receptors (RYRs) and $InsP_3Rs$. **Mitsuhiko Ikura** described the ligand-binding

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domain of the $InsP_3R$. $InsP_3$ was found to bind to a cleft located between an α-domain (a C-terminal region composed of α helices) and a β-domain that consists of a β-trefoil domain. This cleft contains a cluster of lysine and arginine residues that interact with the three phosphoryl groups of $InsP_3$. When $InsP_3$ binds, it clamps together the α- and β-domains to make a more rigid structure. The surface of the β-domain also contains acidic residues that may function to bind the Ca^{2+} that cooperates with $InsP_3$ to induce the conformational change necessary for channel opening. It was proposed that $InsP_3$ binds first to induce an intermediate state that is then converted to the open state by Ca^{2+}.

An important feature of both the $InsP_3Rs$ and the RYRs is that their activity can be finely tuned by a host of associated factors. **Xander Wehrens** described how FKBP12.6, now known as calstabin 2, functions normally to stabilize the RYR2s in cardiac cells. When serine 2809 on RYR2 is phosphorylated by protein kinase A, calstabin2 dissociates from the channel resulting in an increased open probability and sensitivity to Ca^{2+}-dependent activation. This modulatory pathway becomes particularly relevant during heart failure when hyperactivity of the adrenergic signalling pathway results in RYR2 hyperphosphorylation and release of calstabin2. The uncontrolled leak of Ca^{2+} contributes to the high diastolic levels of Ca^{2+} while the reduced SR Ca^{2+} load accounts for the weak Ca^{2+} transients. These observations are relevant to catecholaminergic polymorphic ventricular tachycardia (CPVT), which is a genetic disease where many of the mutations have been mapped to RYR2. The risk of catecholaminergic-induced arrhythmias may result from an increased release of calstabin2 and the unstable gating behaviour described earlier. **Wehrens** described some novel therapeutic agents that may act to enhance the affinity of calstabin2 thereby protecting the heart from the fatal arrhythmias that cause sudden heart death.

3. The geography of Ca^{2+} signalling

Alexei Tepikin referred to the "geography of Ca^{2+} signals" to draw attention to the fact that this signalling system often has a particularly precise spatial organization. He illustrated this point by describing how the release of Ca^{2+} by the $InsP_3Rs$ in the apical region of pancreatic acinar cells is confined by a mitochondrial firewall. At higher agonist concentrations, this firewall is breached and Ca^{2+} breaks out and spreads throughout the cell as a global signal. This local to global transition can alter the distribution of calmodulin (CaM), which concentrates in the apical region at low agonist doses but enters the nucleus when Ca^{2+} spreads globally. **Michael Whitaker** also described changes in the spatial organization of CaM that occurred during mitosis in sea urchin eggs. It localizes around the nucleus at nuclear envelope breakdown (NEBD) and then concentrates at the two spindle poles later in mitosis.

The spatial properties of Ca^{2+} signals are often determined by a precise organization of the Ca^{2+} signalling components such as the entry and release channels. For example, **Leon Lagnado** described how the voltage-operated channels (VOCs) at the synaptic endings of bipolar ganglion neurons are arranged in close proximity to the synaptic vesicles. He described his studies using total internal reflection fluorescence microscopy (TIRFM) to characterize the relationship between the VOCs and the release sites. Ca^{2+} entering through a VOC creates a microdomain that activates vesicles within a radius of approximately 200 nm. **Marie-France Bader** described the geographical arrangement of the many signalling components responsible for triggering exocytosis in chromaffin cells. Some of these components are the sensors that detect the Ca^{2+} pulse whereas others control the process of membrane fusion. **Bader** identified a role for phospholipase D1 (PLD1) that forms phosphatidic acid, which may function to bend the plasma membrane prior to exocytosis. The activation of PLD1 depends upon the small GTPase Arf6, located in the vesicle membrane, binding to ARNO attached to the plasma membrane. These signalling complexes appear to be organized at the sites of exocytosis by lipid rafts that may be stabilized by annexin-2. During the process of exocytosis, therefore, there is a close association between Ca^{2+} entry sites and the downstream effector system that drives vesicle fusion.

A similar close association between Ca^{2+} release and downstream effectors is evident during excitation–contraction coupling in cardiac cells where the SR containing the RYR2s is intimately associated with the myofibrils. **Vincenzo Sorrentino** described some of the proteins responsible for the assembly and stabilization of this signalling organelle. For example, proteins such as the mitsugumins and junctophilins ensure that the RYR2 channels within the junctional SR are lined up facing the VOCs in the T-tubule membrane. The Ca^{2+} spark generated by these RYRs at each junctional zone diffuses a short distance onto the myofibrils to activate contraction. During the recovery process, the interconnected tubular SR network that surrounds each myofibril rapidly sequesters Ca^{2+}. This close association is achieved through a molecular link between an isoform of ankyrin-1, which is embedded in the SR membrane, and the large protein obscurin that is associated with the myofibrils.

Frank Wuytack described another example of a Ca^{2+} signalling component having a precise cellular location, in this case a Ca^{2+} pump. The secretory pathway Ca^{2+}-ATPases (SPCA1 and 2) are located in the Golgi. The SPCA2 isoform has a restricted tissue distribution whereas the SPCA1 is ubiquitous and functions as a housekeeping gene to pump Ca^{2+} from the cytoplasm into the Golgi where it is required for efficient protein processing. Mutations of SPCA1 that result in defects in this pump are responsible for Hailey–Hailey disease, which is characterized by keratinous skin lesions.

4. Mitochondria and Ca^{2+} signalling

The role of the mitochondria in sculpting Ca^{2+} signals in the pancreas was described earlier, but this is just one of its many roles in cellular Ca^{2+} homeostasis and function. **Rod O'Connor** described how mitochondria functioning in their normal cellular context are capable of rapidly sequestering Ca^{2+} as soon as it reaches a critical level of approximately 330 nM; this is very much lower than the 10 μM value determined previously using isolated mitochondria. These observations mean that mitochondria are constantly in play during Ca^{2+} signalling because there is a constant ebb and flow of Ca^{2+} through the mitochondrial matrix whenever there is an increase in cytosolic Ca^{2+}. Using a targeted luciferase probe, **Rosario Rizzuto** confirmed that such an elevation in Ca^{2+} functions to increase ATP formation. **Rizzuto** also described how abnormally high elevations in Ca^{2+} signalling could impinge on mitochondrial metabolism to trigger the onset of apoptosis. Such an excessive uptake of Ca^{2+} becomes deleterious if it activates the permeability transition pore (PTP) resulting in the release of cytochrome *c* and the onset of apoptosis. While there is some agreement that Bcl-2 may act to reduce this mitochondrial uptake of Ca^{2+} by limiting the amount of Ca^{2+} coming from the ER, there are differing opinions regarding how Bcl-2 might regulate ER Ca^{2+} release. **Rizzuto** described his studies on the overexpression of Bcl-2 that suggested that it might act to reduce the level of Ca^{2+} within the lumen of the ER. However, others consider that Bcl-2 acts on the $InsP_3R$ to reduce the release of Ca^{2+} [1]. Whatever the mechanism turns out to be, the interesting possibility remains that Bcl-2 may act to inhibit apoptosis by reducing the amount of Ca^{2+} circulating through the mitochondria.

Another important feature to emerge about mitochondria is that their function may be carefully regulated through a variety of mechanisms. **Rizzuto** described a number of mechanisms for regulating mitochondrial function including the action of different PKC isoforms, Shc and through the activity of proteins that regulate fusion (mitofusins 1 and 2) and fission (dynamin related protein 1). **Pontus Aspenstrom** also described some novel Mitochondrial Rho (MIRO 1 and 2) isoforms that might play a role in the mitochondrial fission/fusion processes. Expression of a constitutively active MIRO1 caused the mitochondria to aggregate and there was an increase in apoptosis. In addition to controlling apoptosis in its host cell, mitochondria may also exert effects on neighbouring cells. **Michael Duchen** described how astrocytes respond to β-amyloid by generating both chaotic Ca^{2+} transients and an increase in free radicals that combine to trigger neurotoxic effects on neighbouring neurons. Just how this interaction is carried out is unclear, but the mitochondria may play a role in this unusual form of intercellular communication. For example, the signals generated in the astrocyte may interfere with the ability of their mitochondria to synthesize and transfer GSH to the neurons, which is critical for their survival when they experience oxidative stress.

5. Ca^{2+} sensors

Cells contain a large number of Ca^{2+}-binding proteins with variable functions. Some, such as parvalbumin and calbindin, function as buffers, which did not feature much at this meeting. However, there was considerable discussion on those proteins that mediate the signalling function of Ca^{2+} and are often referred to as "Ca^{2+} sensors". During the course of the meeting, it became apparent that it would be desirable to have a definition of a Ca^{2+} sensor. If one considers a classical Ca^{2+} sensor such as troponin c, there is a clear-cut signalling pathway: when the sensor binds Ca^{2+} it undergoes a conformational change that then activates the actin/myosin interaction to induce contraction. It seems that relatively few of the Ca^{2+}-binding proteins can be considered to be Ca^{2+} sensors based on this rather minimal definition. Another example of a Ca^{2+} sensor is calmodulin (CaM), which resembles troponin c but has a much more general action in that it controls the activity of many different processes. There appears to be less information on the signalling function of some of the other putative Ca^{2+} sensors such as the annexins and the S100 proteins. **Reginald Morgan** discussed the evolutionary history of these various Ca^{2+}-binding proteins. He used the annexins to map out the evolutionary development of this protein family that has undergone remarkable structural diversity. **Morgan** stressed that such an analysis may help to uncover the physiological role of these somewhat enigmatic and highly diversified Ca^{2+}-binding protein families such as the annexins and the S100 proteins. On the other hand, **Mitsuhiko Ikura** mentioned that CaM was one of the most highly conserved proteins coming 5th in the "genome conservation competition" after histone H4, histone H3, actin B and ubiquitin.

The way in which CaM functions as a Ca^{2+} sensor is featured significantly during the course of the meeting. Its translocation and concentration in different locations within the cell were described earlier. **Walter Chazin** discussed the way in which this sensor selects its targets. He discussed protein architecture and the conformational changes that are induced by Ca^{2+} and the nature of the surface that binds to its targets. **Mitsuhiko Ikura** drew attention to the fact that the target-binding site is a hydrophobic pocket containing numerous methionine residues. Since the latter are highly flexible, they create a binding pocket that is capable of adapting and interacting with different downstream effectors.

One of the major downstream effectors of CaM is the Ca^{2+}/calmodulin-dependent kinase II (CAMKII), which acts on multiple targets located in different cellular locations. In the case of cardiac cells, **John Dedman**

generated transgenic mice expressing in a cardiac specific manner CAMKII peptide inhibitors targeted to either the SR or to the nucleus. The SR targeted inhibitor reduced the phosphorylation of phospholamban resulting in a decrease in the uptake of Ca^{2+} and the onset of cardiac hypertrophy. By contrast, the nuclear-targeted inhibitor caused a decrease in the phosphorylation of the transcription factor CREB and induced the opposite effect of cardiac hypotrophy. This demonstration that CAMKII has markedly different actions depending on its cellular site of action is another example of the importance of understanding the geography of Ca^{2+} signalling.

One of the important functions of CaM is to operate various feedback loops, such as the stimulation of adenylyl cyclase by Ca^{2+} to produce cyclic AMP. This endogenous signalling pathway is usually carefully regulated, but this control breaks down during infection with *B. anthracis*. **Wei-Jan Tang** described how CaM functions to activate the edema factor, which is one of the anthrax toxins responsible for causing death from respiratory failure. Edema factor is a CaM-dependent adenylyl cyclase that produces large amounts of cyclic AMP that causes the marked edema that occurs following infection.

Another Ca^{2+} sensor with multiple downstream targets is neuronal Ca^{2+} sensor protein (NCS-1), which was described by **Bob Burgoyne**. Unlike hippocalcin, which is another member of the NCS family that has a Ca^{2+} myristoyl switch enabling it to associate with membranes in a Ca^{2+}-dependent manner, NCS-1 is already associated with either the Golgi or the plasma membrane. These different associations seem to depend on a lipid-binding domain in the N-terminal region of the protein. The ways in which NCS-1 and other members of the family are activated seem also to depend upon the spatial organization of Ca^{2+} signals.

For the Ca^{2+} sensors, such as troponin c, CaM and NCS-1, there is a reasonable understanding of how Ca^{2+} acts through these proteins to activate downstream effectors. However, this is not always the case for some of the other putative Ca^{2+} sensors such as the annexins and the S100 proteins. While it is clear that they perform important functions within the cell, what is lacking is whether these functions are part of a Ca^{2+} signalling pathway. For example, although Ca^{2+} induces the membrane translocation of many of the annexins, it is not clear how this relates to their subsequent function. For example, **Volker Gerke** described how annexin-2 interacts with S100A10 to function in the operation of the recycling endosome during endocytic cycling. But how does Ca^{2+} contribute to the control process other than to induce the initial translocation? It would be interesting to determine the precise relationship between Ca^{2+} signalling and annexin function because there is growing evidence that they function in the onset of cancer as was emphasised by **Harvey Pollard**. He illustrated this by describing a role for annexin-7, but the picture is complicated by the fact that its level is low in prostate cancer but high in aggressive forms of breast cancer. In the case of prostate cancer, it appears that it may function as a tumour suppressor perhaps acting through cyclin E.

The S100 proteins have also been implicated in the action of Ca^{2+}, but like the annexins, there appears to be little information about how Ca^{2+} contributes to their activation of downstream effectors. For example, does Ca^{2+} play any role in the way in which S100B acts to inhibit the tumour suppressor p53 as was described by **David Weber**? Such a possibility is particularly interesting because S100B is elevated in primary malignant melanomas and may act to suppress the expression of p53. It would be interesting to know whether Ca^{2+} signalling plays any role in the S100B-dependent gene transcriptional events that appear to regulate cell proliferation. This becomes all the more interesting because **Stéphanie Thebault** reported the α-adrenergic stimulation was able to induce Ca^{2+} oscillations and the proliferation of primary human prostate cancer cells.

6. Ca^{2+} regulation of development

Signal Ca^{2+} controls a wide range of cellular processes. Many of these concern the way Ca^{2+} regulates many development events including fertilization, embryogenesis and cellular differentiation. **Michael Whitaker** described how speract induces Ca^{2+} signals to control sea urchin sperm motility and chemotaxis. Like many other Ca^{2+} signalling processes, these developmental events are driven by oscillations in intracellular Ca^{2+}. Once the sperm finds the egg and fertilization occurs, Ca^{2+} is used again, this time to drive cell cycle events. This process was examined in detail in *Drosophila* embryos where Ca^{2+} has an important role in both cell cycle entry and exit. **John Carroll** described a similar role for Ca^{2+} in driving the cell cycle forward during fertilization in mammalian eggs. The regular spikes that occur after fertilization are driven by a novel phospholipase C zeta (PLCζ), which is injected into the egg by the sperm. The oscillations cease when the PLCζ is sequestered into the pronuclei once the nuclear membranes are formed. Many hours later, the pronuclei break down at the time of the first mitosis and the PLCζ is released back into the cytoplasm where it begins to generate the $InsP_3$ that induces spontaneous spikes during the mitotic events.

Peter Mobbs described another example in the developing retina where waves of Ca^{2+} play a role in driving mitosis in cells located in the ventral zone. These retinal Ca^{2+} waves appear to be driven by ATP released from the overlying retinal pigment epithelium. If the supply of ATP is removed by taking away the pigment epithelium, mitosis is greatly reduced but can be restored by treating the ventral zone cells with UTP/ATP. The way in which ATP is released from the retinal pigment layer is complicated but it seems to depend on waves of Ca^{2+} that function to release ATP from connexin hemi-channels.

Howard Bayliss described a role for $InsP_3$-induced Ca^{2+} mobilization in many of the developmental events that occur following fertilization of *C. elegans*. Various genetic mutations, including the expression of an $InsP_3$ sponge to buffer the intracellular level of this messenger, were found to disrupt a number of developmental processes during embryogenesis such as cytokinesis, cell fate decisions and morphogenesis. One of the decisive defects was a down-regulation of the transcriptional events that drive the differentiation of a gut cell lineage derived from a single E founder cell. The characteristics of the Ca^{2+} signals that drive differentiation in *C. elegans* have not been characterized. However, in the case of mammalian development, it is clear that oscillations in the intracellular level of Ca^{2+} drive certain forms of differentiation. For example, **Catherine Leclerc** described how the process of tubulogenesis in the developing *Xenopus* kidney is preceded by the appearance of Ca^{2+} oscillations.

This role of Ca^{2+} in driving differentiation displays a remarkable degree of subtlety during development of the nervous system. For example, **Nick Spitzer** showed how the frequency of Ca^{2+} oscillation determined the type of neurotransmitter expressed in different neuronal cell types. The mechanism controlling this process of neuronal differentiation displays homeostatic properties. Suppression of oscillations increased the number of neurons expressing excitatory transmitters such as glutamate and acetylcholine, whereas the opposite occurs for the inhibitory transmitters glycine and GABA that appeared when oscillation frequency was increased. Once neurons have differentiated into specific types, they have to wire up to each other to form functional circuits, which is the final process of brain development. **Rachel Wong** described how Ca^{2+} has a specific role to play in this wiring-up process within the developing retina. Both global and local Ca^{2+} signals were recorded in the developing retina. The global signals appear as spiral waves that engulf large populations of neurons. On the other hand, the local signals are recorded in the dendrites during synapse formation as neurons begin to make contacts with each other. For example, when the axonal terminal of an amacrine cell makes contact with the dendrite of a bipolar cell, it generates a local Ca^{2+} transient. One hour later, the segment of the bipolar cell contacted by the amacrine cell begins to generate a series of Ca^{2+} transients. These Ca^{2+} transients play a role in stabilizing the new synaptic connections. In effect, the local pulsing of Ca^{2+} functions as a spot weld to stabilize the newly formed synapses of the developing nervous system.

7. Summary

It is clear from this meeting that Ca^{2+} signalling is an active and vibrant field of research where there are many unsolved problems. There is still great uncertainty about how agonists stimulate Ca^{2+} entry and what the role of the TRP channels might be. On the other hand, there is general agreement about the role of the $InsP_3Rs$ and the RYRs in the release of internal Ca^{2+}. With regard to the latter, the questions now being asked concern their structural features and the modulatory roles of their associated proteins. The function of the downstream Ca^{2+} sensors has also seen considerable progress and structural information is now emerging about how sensors such as CaM detect Ca^{2+} and then transmit the information to downstream effectors. However, there still seems to be a lack of understanding about exactly how some of the other sensors are responding to Ca^{2+} transients to control downstream effectors. This is a fascinating area given that many of these Ca^{2+}-binding proteins, such as the annexins and the S100 proteins, seem to play such an essential role in cell proliferation. There is still much to learn and the European Calcium Society and especially the conference organiser Steve Moss should be congratulated for putting together a conference programme that brought into such sharp focus both the achievements and many of the future problems that lie ahead of us in the field of Ca^{2+} signalling.

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Reference

[1] R. Chen, et al., J. Cell Biol. 166 (2004) 193–203.