9th Meeting
of the European Calcium Society

Ecole Supérieure de Biotechnologie, Strasbourg

19-22 July 2006

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Wednesday, 19 July 2006 pm

13.00 - 19.00	**Registration**	
19.00 - 20.00 **Chair : V. Gerke (Munster,Germany)**	**M. Berridge (Cambridge, UK)**	**Calcium signaling in health and disease**

20.00 - 23.00 : WELCOME RECEPTION

Thursday, 20 July 2006

SESSION 1 : Ca^{2+}-binding proteins (chemistry, therapeutic targets, evolution)

Chair : C.B. Klee (Bethesda, USA)

8.30 – 9.30 **Keynote lecture**	**R.P.J. Williams (Oxford, UK)**	**The evolution of calcium functions**
9.30 - 10.00	**R.O. Morgan (Oviedo, Spain)**	**Deciphering function and mechanism of calcium-binding proteins from their evolutionary imprint**
10.00 – 10.30	**D. Weber (Baltimore, USA)**	**S100B is a calcium-activated switch that turns off the tumor suppressor protein p53 in malignant melanoma**

10.30 – 11.00 : BREAK

11.00 – 11.15	**A. Galichet (Zurich, Switzerland)**	**The RAGE receptor and the S100 proteins in brain pathology**
11.15 – 11.30	**Y. Izumi, Yamagata, Japan**	**Interaction of calmodulin with the cytoplasmic domain of a transmembrane GP41 : a novel binding mode of calmodulin molecular recognition**
11.30 – 12.00	**A. Vedeler (Bergen, Norway)**	**Annexin A2 as a mRNA-binding protein**

12.00 – 15.00 : LUNCH / <u>POSTER SESSION</u>

SESSION 2 : Gene expression and calcium signaling

Chair : M. Moreau (Toulouse, France)

15.00 – 16.00 **Keynote lecture**	**P. Chambon (Strasbourg, France)**	**Genetic dissection of the function of RXRs and their heterodimeric partners in homeostasis and disease of adult mouse epidermis**
16.00 – 16.30	**C. Leclerc (Toulouse, France)**	**Calcium transients and calcium signaling during early neurogenesis in the amphibian embryo Xenopus laevis**
16.30 – 17.00	**R. Ranjeva (Castanet-Tolosan, France)**	**Cytosolic and nuclear calcium signaling in plant cells : regulation and biological implications**
17.00 – 17.15	**H. Suzuki (Nagoya, Japan)**	**Analyses of calcium-dependent interactions between ALG-2 and its binding partners involved in membrane trafficking**

17.15 – 17.45 : BREAK

17.45 – 18.00	**M. Savignac (Madrid, Spain)**	**Role of dream, a transcriptionnal repressor regulated by calcium, in immune system**
18.00 – 18.30	**A. Hetherington (Lancaster, UK)**	**The guard cell calcium-based signaling network**
18.30 – 19.00	**A. Miller (Hong Kong)**	**Ca^{2+} signaling during early embryonic patterning in zebrafish**

20.00 : RECEPTION AT THE CITY HALL

FREE EVENING

Friday, 21 July 2006

SESSION 3 : Calcium signaling and trafficking

Chair : R. Donato (Perugia, Italy)

8.30 – 9.00	**M.F. Bader (Strasbourg, France)**	**Phospholipase D1 in calcium-regulated exocytosis : a checkpoint for monomeric GTPases**
9.00 – 9.30	**N. Brose (Goettingen, Germany)**	**Calcium-dependent regulation of synaptic vesicle priming and synaptic short-term plasticity**
9.30 – 10.00	**F. Wuytack (Leuven, Belgium)**	**The SPCA-type of Ca2+ transport ATPases**

10.00 – 10.30 : BREAK

SESSION 4 : Calcium channels

Chair : C. Heizmann (Zurich, Switzerland)

10.30 – 11.00	**J. Putney (NC, USA)**	**Modes of activation of CRAC and TRPC channels**
11.00 – 11.30	**D.L. Gill (Baltimore, USA)**	**STIM proteins - mediators and regulators of store-operated channels**
11.30 – 12.00	**P. Lory (Montpellier, France)**	**Voltage-gated calcium channels in genetic diseases**

12.00 – 15.00 : LUNCH / <u>POSTER SESSION</u>

14.00 – 15.00	**GENERAL ECS ASSEMBLY**

SESSION 5 : Calcium binding proteins and pathophysiology

Chair : R. Pochet (Brussels, Belgium)

15.00 – 16.00 Keynote lecture	**D.M. Watterson (Chicago, USA)**	**Calmodulin-dependent kinases as therapeutic targets**
16.00 – 16.30	**J. Roth (Munster, Germany)**	**Inflammatory activities of myeloid related protein 8 (MRP8, S100A8) and MRP14 (S100A9)**
16.30 – 16.50	**R.A. Schulz (Houston, USA)**	**Molecular basis of calcineurin function in Drosophila muscle development**

16.50 – 17.00	**F.F. Dai (Toronto, Canada)**	**Visinin-like-protein-1 (VILIP-1) is expressed in pancreatic β-cells and regulates insulin secretion and insulin gene transcription**
17.00 – 17.15	**R. Donato (Perugia, Italy)**	**S100B/rage-dependent activation of Microglia : stimulation of COX-2 expression and TNF-α release**
17.15 – 17.30	**J.R. Dedman (Cincinatti, USA)**	**Nuclear Ca^{2+}/calmodulin-dependent protein kinase II in the murine heart**
17.30 – 18.00	**J. Kuznicki (Warsaw, Poland)**	**Biochemical features of presenilins and their involvement in Ca^{2+} signaling of lympho-blasts from Alzheimer's disease patients**

CONFERENCE BANQUET

Saturday, 22 July 2006

SESSION 6 : Systems Biology (signaling pathways, technical aspects, nanotechnology, virtual cell)

Chair : S.E. Moss (London, UK)

9.00 – 10.00 Keynote lecture	**C. Klee (Bethesda, USA)**	**From calcium calmodulin to gene expression**
10.00 – 10.30	**H.L. Roderick (Cambridge, UK)**	**Calcium release through inositol 1,4,5-trisphosphate receptors is required for the induction of cardiac hypertrophy**

10.30 – 11.00 : BREAK

11.00 – 11.30	**A.H. Guse (Hamburg, Germany)**	**NAADP : from second messenger to therapy**
11.30 – 11.45	**V. Sorrentino (Siena, Italy)**	**A FRAP-based analysis of the assembly and dynamics of sarcoplasmic reticulum proteins in skeletal muscle cell**
11.45 – 12.10	**J.J. Yang (Atlanta, USA)**	**Rational design and prediction of calcium binding proteins**
12.10 - 12.30	**V. Gerke (Munster, Germany)**	**CLOSING REMARKS**

12.30 : LUNCH BASKET and END OF THE MEETING

ABSTRACTS

Opening lecture

CALCIUM SIGNALING IN HEALTH AND DISEASE

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Calcium ($Ca^{2+)}$ is a highly versatile intracellular signal capable of regulating many different processes. To achieve this versatility, the signaling system operates in many different spatial and temporal modes thus enabling it to function over a wide dynamic range. At any moment in time, the level of intracellular Ca^{2+} is determined by a balance between the ON reactions that introduce Ca^{2+} into the cytoplasm and the OFF reactions during which this signal is removed through the combined action of buffers, pumps and exchangers.

Cells have access to a very extensive Ca^{2+} signaling toolkit from which each cell type expresses a unique set of components to create Ca^{2+} signaling systems with widely different spatial and temporal properties. Spatial properties are particularly relevant for fast responses where components of the ON reactions and their downstream effectors are closely associated. This spatial contiguity is less apparent for the slower responses such as gene transcription, fertilization and cell proliferation where Ca^{2+} signals tend to operate more globally and where temporal properties of signaling become increasingly important with signaling represented as repetitive Ca^{2+} transients and waves.

Such Ca^{2+} signaling systems are not fixed in stone, but are constantly being remodelled to adapt to changing circumstances to ensure that each specific cell type continues to deliver the Ca^{2+} signals that characterizes its unique function. It seems that Ca^{2+} itself plays a critical role in this internal assessment mechanism by remodelling its own signaling pathway. A number of important disease states (hypertension, congestive heart failure, manic depressive illness, Alzheimer's disease) may result from abnormal remodelling of Ca^{2+} signaling systems. A good example is congestive heart failure, a major cause of human morbidity and mortality, which is controlled by a number of signaling pathways of which Ca^{2+} seems to play a prominent role.

Session 1 : Ca^{2+} binding proteins

THE EVOLUTION OF CALCIUM FUNCTIONS

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The earliest functions of calcium which we can speculate about indicate that it could have had functions outside cells in stabilising external structures and possibly in activating digestive proteins in the environment. These use are still seen in many bacteria. They require an external calcium concentration of about millimolar and the structures are such that they do not bind magnesium. Calcium was excluded from the cytoplasm to avoid competition with magnesium and the precipitation of anions. It would appear that the value of calcium sites may well have been a precursor of the oxygen generating centre of photosystem 2 much of it replaced by manganese. Oxygen led to the evolution of eukaryotes and from that time less than 2 billion years ago the value of calcium has increased as a signaling ion.Proteins with novel binding sites and much higher binding constants appeared in the cytoplasm while new sites of lower stability are found in the reticula in a series of developments.

Coordination of activity in eukaryotes has to between vesicle, organelle and the cytoplasm as well as between external events and internal responses. Probable the calcium ion dominates all the communication in the single cell eukaryotes. Extension to multi-cellular eukaryotes required a message system linked to these calcium functions between cells. These messengers are commonly small organic molecules often released by calcium action in one cell and causing calcium input at another. The ramification of this message system came to include the nerves and brains of animals but now the fastest of all ionic transmission was also incorporated - the use of sodium and potassium gradients. It is amazing that such a simple ion could become responsible for so much because it had to be rejected by the earliest cells.

DECIPHERING FUNCTION AND MECHANISM OF CALCIUM-BINDING PROTEINS FROM THEIR EVOLUTIONARY IMPRINT

R.O. Morgan and M.P. Fernandez

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The diversity and relationships between structure and function among calcium-binding proteins can be assessed in a systematic manner by employing computational biology techniques in comparative genomics. A vital resource for such studies is the evolutionary history of gene families because it provides the phylogenetic framework upon which to examine patterns and processes of structural change that have been imposed by functional constraints during species evolution. Subsequent refinements include the subclassification of distinct structure-function models based on site-specific conservation and divergence, the elaboration of probabilistic hidden Markov models to define active sites with molecular profiles, and the construction of 3D protein models to convey the evolutionary information. We have thus charted the evolutionary history of the 3 major groups of calcium-binding domains (viz. EF-hand, annexin and C2) in selected protein families to determine the nature, rate and extent of structural changes they have undergone. The aims were to define the site-specific variation in each domain type and to identify significant evolutionary events that coincided with major adaptations or divergence in their structure and function. In this way, we hoped to better comprehend the full range of protein structures involved in calcium regulation and to scrutinize specific models in an evolutionary context to decipher the molecular basis of their function. A focus on EF-hand evolution used both phylogenetic and clustering algorithms to characterize structural variation among thousands of homologs from public databases, including computationally assembled sequences. Distinctive patterns of change were evident in comparisons of individual families such as calmodulin, excalibur, multiEF-hand, and S100. Accessory sites near the calcium-coordinating residues, the susceptibility to conformational change, and the domain architecture in relation to other functional domains or target receptors all contribute the individuality of EF-hand function in different proteins. The same variables can be identified in annexin and C2-containing proteins but in different measures as defined by computational analyses. Interestingly, the convergence of calcium-binding proteins at cell membranes may coincide with the formation of multimeric and multidomain complexes and their evolutionary interdependence of function, exemplified by S100-annexin interactions. A novel hypothesis will be presented to account for the prevalence of annexin KGD domains by their possible interaction with C2 domains to ensure membrane targeting and diversify target selection. Other related topics will cover the transformation of calcium-binding proteins during the emergence of vertebrates and the prediction of calciopathies from the HapMap project.

Reference:
RO Morgan, S Martin-Almedina, JM Iglesias, MI Gonzalez-Florez, MP Fernandez. Evolutionary perspective on annexin calcium-binding proteins. Biochim Biophys Acta 1742: 133-140 (2004).

Keywords: Computational biology, evolution, functional determinants, membrane binding, receptor docking, structural motifs

S100B IS A CALCIUM-ACTIVATED SWITCH THAT TURNS OFF THE TUMOR SUPPRESSOR PROTEIN p53 IN MALIGNANT MELANOMA

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Elevated S100B protein is a prognostic marker for assessing disease progression, recurrence, and metastatic potential in patients with malignant melanoma (*1*), the 5th and 7th most common cancer among men and women, respectively, with more than 53,600 new occurrences per year (*2*). While p53 is often mutated in human cancers (*3*), it is nearly always wild-type (wt) in malignant melanoma (*4*). However, we and others have discovered that S100B is not just a clinical marker, but rather, it binds to p53 (*5, 6*), promotes p53 protein degradation, and inhibits its tumor suppression function in malignant melanoma (*5-8*). Furthermore, inhibiting S100B in malignant melanoma (via siRNA^{S100B}) restores wt p53 protein levels and its transcriptional activation/apoptosis activities (*5, 9, 10*). Thus, elevated S100B levels in cancers such as malignant melanoma, renal cell carcinomas (*11*), and some forms of leukemia (*12*) contribute to cellular proliferation by down-regulating wild-type p53. A discussion regarding the interaction between S100B and p53 *in vitro* and in cancer cell and whether it is regulated by divalent cations (Ca^{2+}, Zn^{2+}) and/or by p53 phosphorylation will be discussed and compared to results from other S100-target protein complexes. Because S100 proteins are elevated in nearly every type of human cancer, a long-range goal is to incorporate what we learn here to tailor small molecule inhibitors that specifically block S100 protein(s), such as S100B, that down-regulate p53.

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Keywords: S100B, p53, disease, melanoma, Zn2+ P18, Ezrin, S100P P20 calmodulin

THE RAGE RECEPTOR AND THE S100 PROTEINS IN BRAIN PATHOLOGY

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The Receptor for Advanced Glycation End products (RAGE) is a member of the immunoglobulin-like cell surface receptor superfamily. It contains an extracellular domain consisting of three immunoglobulin-like regions, one "V"-type followed by two "C"-type, a single transmembrane-spanning domain and a 43-amino acid cytosolic tail. RAGE is widely expressed in all human organs and several RAGE isoforms are generated by alternative splicing.

One major characteristic of RAGE is its ability to interact with a broad repertoire of ligands, including advanced glycation end-products (AGEs), amphoterin, amyloid peptide, and S100 proteins. S100 proteins are small calcium binding proteins presenting tissue- and cell-specific expression patterns and forming homo, heterodimers and even oligomers. These proteins possess intracellular functions such as calcium homeostasis, protein phosphorylation, interaction with cytoskeleton constituents, cell cycle regulation, cell growth and differentiation. In addition, S100B, S100A1, S100A4, S100A8, S100A9 and S100A12 are secreted and act in a cytokine-like manner. Furthermore, S100 proteins are associated with various human pathologies such as Alzheimer's disease (AD), cancer, inflammation or cardiomyopathies.

We investigated the physical interaction of members of the S100 protein family with RAGE by Surface Plasmon Resonance and found that S100B, S100A1, S100A4, S100A6 and S100A12 bind to RAGE with □M affinity. Because S100B, S100A1 and S100A6 expression is altered in AD, we evaluated the influence of these proteins on human SH-SY5Y neuroblastoma cells as well as the involvement of RAGE in signal transduction following RAGE-S100 protein interaction. Interestingly, the three S100 proteins tested affect cell proliferation and differentiation in a dose-dependent manner. Furthermore, blockade approaches, using sRAGE, the extracellular part of the RAGE receptor, or specific RAGE antibodies, successfully revert the biological effects of the S100 proteins. In addition, these biological responses are dependent on the activation of several signaling pathways.

Altogether our analyses reveal the high complexity of the RAGE network and highly suggest that the S100 protein family members could exert their functions by interacting with RAGE.

Keywords: RAGE, S100A12, Alzheimer

INTERACTION OF CALMODULIN WITH THE CYTOPLASMIC DOMAIN OF A TRANSMEMBRANE GP41: A NOVEL BINDING MODE OF CALMODULIN MOLECULAR RECOGNITION

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Small-angle X-ray scattering using synchrotron radiation as an X-ray source has been employed to analyze the interaction of calcium-bound calmodulin (Ca^{2+}/CaM) with a lentivirus lytic peptide type 1 (LLP1) derived from the cytoplasmic tail of HIV-1 transmembrane glycoprotein, gp41. The synthetic peptide homologues of LLP1 were derived from three species of the glycoprotein: ENV_HV1A2, ENV_HV1B1, and ENV_HV1H2. The results indicate that the solubility of the peptides widely changes depending on a deletion of the C-terminal CaM-binding domain. Ca^{2+}/CaM binds LLP1 with a deletion of three or ten amino acid residues at a molar ratio of 1:1, indicating that the CaM-binding site locates on the sequence with the deletion of ten residues. The Ca^{2+}/CaM/LLP1 complex adopts almost the same globular structure as that previously observed in the Ca^{2+}/CaM/MLCK peptide complex[1)] but no amino acid sequence in the LLP1s match to the database[2)]. Furthermore, Ca^{2+}/CaM binds a peptide with the opposite sequence and adopts almost the same globular structure as that previously observed[1)], indicating that the LLP1 binds Ca^{2+}/CaM in the opposite orientation to that previously observed[1)]. At the two opposite outlets of the hydrophobic channel composed by two domains of CaM are clusters of acidic residues that are asymmetric in size.

The LLP1 that interacts with this channel possess complementary polarity created by a cluster of basic residues in the amino acid sequences. The basic cluster of the MLCK peptide is located at the N-terminal side of the CaM-binding region, while that of the LLP1s is located at the C-terminal side. Thus, the results indicate that the location of a basic cluster within the CaM-binding region plays an important role in determining the direction of its binding with respect to CaM domains, as previously suggested[3)]. Threading suggest that the LLP1 segment may fold back on another LLP segment (LLP2) in antiparallel fashion[4)].

Taken together, we speculate that the Ca^{2+}/CaM-binding to the cytoplasmic tail induces a large change in the tertiary structure.

The results of these studies show that the binding mode of Ca^{2+}/CaM molecular recognition is well preserved despite of the sequence variation of three species of gp41, suggesting that this region of the glycoprotein is important to viral replication.

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Keywords: HIV-1; gp41; LLP1; calmodulin; SR-SAXS

ANNEXIN A2 AS AN mRNA-BINDING PROTEIN

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Annexin A2 is a multifunctional protein implicated in cellular processes such as signal transduction, membrane trafficking, mRNA transport and membrane/cytoskeleton interactions. The protein also appears to have a key role in cell transformation and its expression is modulated in numerous cancers. Annexin A2 binds different intracellular and extracellular ligands and is subjected to numerous post-translational modifications. In addition to Ca^{2+}, its core domain binds F-actin, 14.3.3 regulatory proteins, cholesterol, phospholipids and mRNA intracellularly while p11, a member of the S100 family, binds to its N-terminal tail. Extracellularly, Annexin A2 binds heparin and appears to be a cellular receptor for t-PA and plasminogen. In eukaryotic cells, RNA-binding proteins regulate the activity, abundance, and stability of both translationally active and non-active RNAs present in mRNP complexes. RNA-binding proteins can organise mRNAs into structurally and functionally related subsets, thus facilitating the coordinate localisation and production of proteins necessary for complex cellular processes. We have shown that Annexin A2 interacts specifically with mRNAs translated on cytoskeleton-bound polysomes and it has recently been identified as an RNA-binding protein that interacts with the localisation signal in the 3'untranslated region (3'UTR) of c-*myc* mRNA. Our recent data reveal that Annexin A2 also binds to a specific region of the 3'UTR of its cognate mRNA while it does not associate *in vivo* with β_2-*microglobulin* mRNA translated on membrane-bound polysomes indicating specificity of the interaction. To detect the amino acid residues involved in mRNA-binding we have produced specific mutants of the mRNA-binding domain of Annexin A2 in collaboration with Gilles Travé (Illkirch, France). As another approach, we are currently producing crystals of a complex containing a smaller region of the 3'UTR of *annexin A2* mRNA bound to Annexin A2 protein in collaboration with Robert H. Kretsinger (Charlottesville, USA). Since we have not been able to define a specific consensus secondary RNA structure that is involved in binding to Annexin A2, solving the structure of the complex will provide the molecular details of its interaction with mRNA. Annexin A2 purified from transformed cells is impaired in its ability to bind mRNA, as compared to the protein isolated from the corresponding normal cells. It also displays different degrees of post-translational modifications indicating that specific post-translational modifications modulate the mRNA-Annexin A2 interaction.

Keywords: annexin A2, mRNP complex , mRNA, c-*myc*, mRNA localisation, 3'UTR

THE ROLE OF S100A1 IN CARDIAC ARRHYTHMIAS UNCOVERED BY USING A MOUSE KNOCK-OUT MODEL

[1]Ackermann G., [1]Bonath I., [2]Domenighetti A., [3]Gusev K., [1]Gemperle C., [1]Bosshart C., [1]Murmann P., [1]Kleinert P., [3]Niggli E., [2]Pedrazzini T., and [1]Heizmann C.

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Cardiac arrhythmias can lead to sudden cardiac death, a lethal circulatory collapse, which claims more than 300'000 lives a year in the United States. Prevention of cardiac arrhythmias has been achieved by the implantation of defibrillators and pacemakers in high-risk patients, and drug therapies. However, our understanding of the substrate underlying arrhythmia syndromes is far from being advanced. Over the last decade, it became evident that the character of this disorder is very heterogeneous, that various genetic as well as pathophysiological states can cause arrhythmias and that uniform treatment must be precluded.

The goal of our research is to get insight into the interaction among genetic, structural and functional factors underlying the susceptibility to, and initiation of arrhythmias.

We have generated a transgenic mouse line, which is deficient for the calcium binding protein S100A1, and which shows arrhythmic events and repolarization deficits upon beta-adrenergic stimulation as determined by prolonged QT intervals on the surface electrocardiogram (ECG). On a cellular level, we find a reduced increase in calcium transient amplitudes upon beta-adrenergic stimulation in mutant versus wildtype cardiomyocytes. To understand the molecular mechanisms connected with the observed cardiac arrhythmias, we screened the S100A1-deficient and wildtype transcriptome and proteome for differentially regulated genes and proteins by gene array hybridization and two-dimensional gel electrophoresis, respectively. We have identified several candidate genes/proteins involved in neuro-muscular function, cell signaling and oxidative stress, which may account for the electrical alterations observed in absence of S100A1.

The dissection of the functional and molecular pathways eliciting S100A1-dependent arrhythmias will provide a more comprehensive understanding of the factors that impair normal electrical activity of the heart, and may uncover novel mechanisms of the pathogenesis of sudden cardiac death.

Reference:
Wilde AAM and Bezzina CR (2005) Genetics of cardiac arrhythmias, Heart 91:1352-1358

Keywords: Heart, arrhythmias, long QT, transcriptome, proteome, S100A1

ENGINEERING AND BIOPHYSICAL CHARACTERISATION OF A SOLUBLE MUTANT FORM OF ANNEXIN A2 DOMAIN IV THAT ADOPTS A PARTIALLY FOLDED CONFORMATION

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[2]Laboratoire de RMN & [3]Équipe Oncoprotéine, École Supérieure de Biotechnologie de Strasbourg, Illkirch, France.

The annexins are a family of structurally related globular proteins that bind and promote aggregation of vesicles containing negatively charged phospholipids in a Ca^{2+}-dependent manner (1,2). The four ~75-residue domains (repeats) that constitute the annexin core structure all possess an identical five-α-helix bundle topology, but the physico-chemical properties of the isolated domains are different. Domain IV of the annexins has previously been expressed only as inclusion bodies, resistant to solubilisation (3). Analysis of the conserved, exposed hydrophobic residues of the four annexin domains reveals that domain IV contains the largest number of hydrophobic residues involved in interfacial contacts with the other domains. We designed five constructs of domain IV of annexin A2 in which several interfacial hydrophobic residues were substituted by hydrophilic residues. The mutant domain, in which all fully-exposed hydrophobic interfacial residues were substituted, was isolated as a soluble protein. Circular dichroism (CD) measurements indicate that it harbours a high content of α-helical secondary structure and some tertiary structure. The CD-monitored (λ = 222 nm) thermal melting profile suggests a weak cooperative transition. Nuclear magnetic resonance (^{1}H-^{15}N) correlation spectroscopy reveals heterogeneous line-broadening and an intermediate spectral dispersion. These properties are indicative of a partially folded protein in which some residues are in a fairly structured conformation, whereas others are in an unfolded state. This conclusion is corroborated by 1-anilinonaphthalene-8-sulfonate fluorescence (ANS) analyses. Surface plasmon resonance (SPR) measurements also indicate that this domain retains the binding of known ligands of domain IV in the full-length annexin A2, such as heparin (4) and Ca^{2+} (1,2), although with lower affinities.

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Keywords: annexin A2, heparin, calcium, domain IV, CD, NMR

CHARACTERIZATION OF THE Ca^{2+}-REGULATED S100P-EZRIN INTERACTION

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Members of the ezrin/radexin/moesin (ERM) family and the related tumor supressor merlin belong to the protein 4.1 superfamily. Ezrin, as well as other members of this family, can function as membrane–cytoskeleton linkers with their N-terminal domains containing membrane linking sites and their C-terminal domains harboring F-actin binding sites.
Ezrin can exist in a dormant conformation, in which the membrane and F-actin binding sites are masked through interactions between the N- and C-terminal domains. Activation of ezrin's membrane/cytoskeleton cross linking activity can result from threonine phosphorylation in the C-terminal domain and PIP_2 binding to the N-terminal domain. We identified the EF-hand type Ca^{2+} binding protein S100P as a novel activator of ezrin and that functions directly in response to Ca^{2+} transients. Ca^{2+} dependent S100P binding to ezrin unmasks the F-actin binding site thereby activates the ezrin molecule. This activation does not require C-terminal threonine phosphorylation.
Ezrin's N-terminal domain (N-ERMAD) harbors the S100P binding site which is accessible in the dormant molecule. To precisely map the region involved in ezrin-S100P complex formation, we performed different interaction studies with a series of C-terminally truncated N-ERMAD and S100P derivates.
In addition, we carried out PIP_2 co-sedimentation assays that revealed a competition of S100P with PIP_2 for the same binding motif in ezrin. The S100P-mediated activation of ezrin could resemble the activation via PIP_2 binding.
All S100P expressing tissues and cells analyzed so far also contain ezrin, indicating that the interaction might be of relevance for a number of cells. Since deregulated S100P expression has been linked to tumor cell migration and metastasis results delineating the role of S100P-ezrin complex formation in spontaneous cell migration will be discussed.

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Keywords: Calcium, F-actin, membrane/cytoskeleton linkeage, membrane rafts

The $GABA_A$-RECEPTOR - A NEW MEMBER OF THE INTERCALATED DISCS IN THE HEART

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S100 proteins comprise the largest subgroup within the superfamily of proteins carrying the (Ca2+)-binding EF-hand motif. The members of the S100 protein family consist of 21 low molecular weight (9-13 kDa) proteins that exhibit cell- and tissue-specific expression. Protein S100A1 is most highly expressed in myocardial tissue [1]. In heart failure, a state of insufficient contractile support, S100A1 was found to be reduced [2] whereas in compensated cardiac hypertrophy, a state of enhanced contractile function, the S100A1 level was shown to be increased [3]. It has been shown that S100A1 co-localizes with contractile filaments and proteins of the sarcoplasmic reticulum, which regulate intracellular calcium transport [4]. Furthermore cardiac-restricted overexpression of the Ca^{2+}-binding protein S100A1 led to increased myocardial contractile performance *in vitro* and *in vivo* [5-7]. Therefore S100A1 is considered to be an important regulator of contractility and Ca^{2+} handling in the heart. In order to identify factors associated with S100A1 function we use a mouse model deficient in S100A1 expression, which was generated by disrupting the S100A1 gene by a retroviral integration vector.

Analysis of S100A1-deficient mice by DNA-microarray showed differences in the mRNA-level of the α1 subunit of the $GABA_A$-receptor in 6 month old female hearts. The mRNA level exhibited a 8 fold downregulation, whereas no changes in protein expression could be observed. The $GABA_A$-receptor is well described in the brain where it acts as a ligand gated chloride channel [8, 9]. It is composed of 5 subunits which assemble in about 60% of the cases in a proportion of 2 (α1) : 2 (β2) : 1 (γ2) subunit [10]. Interestingly the α1, β2/3 and γ2 subunits of the $GABA_A$-receptor are expressed in cardiomyocytes at the intercalated discs where they colocalize with β-catenin, a member of the adherens junction. The intercalated discs consist of different components: the adherens junction, the gap junction and the desmosomes [11] and are important for cell-cell communication. Additional experiments will focus on the functional role of the $GABA_A$-receptor in the heart by voltage-clamp experiments, on possible interaction partners and on the transcriptional regulation of the α1 subunit.

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Keywords: calcium, S100A1, heart, intercalated disk, $GABA_A$-receptor

CALMODULIN INTERACTING MOLECULES

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The ability to discover chemical compounds for novel therapeutic targets, is the first critical step in drug discovery programs.
The aim of our work is to develop a screening assay for soluble proteins using fluorescence methods, specially fluorescence polarization/anisotropy (FP) which is widely used in the study of protein-ligand interactions because of its inherent sensitivity, and the fact that they can be implemented at true equilibrium conditions. FP is a very useful tool to study association equilibrium when free- and bound-species implicated in the equilibrium have different rotational speeds.
The calcium ion (Ca^{2+}) is an ubiquitous second messenger that is crucial for the regulation of a wide variety of cellular processes implicating many Ca^{2+}-binding proteins, among which calmodulin (CaM) is the best studied in the eukaryotic cell. CaM binds to a variety of cellular proteins regulating their activities and is thus implicated in many pathological processes of disease such as inflammation, ischemic neuronal death, Alzheimer, Parkinson, cancer, diabetes, cardiomyopathy and changes in behaviors.
We have developed a FP-high-throughput screening (FP-HTS) assay for CaM, using synthetic CaM (SYNCAM)[1] and specific mutants[2, 3]. Screening a fluorescent chemical library composed of about 1400 lissamine-tagged compounds allowed us to retrieve four probes that bind to CaM in a Ca^{2+}-dependent manner. This primary FP-HTS provides us with a tool for screening the Prestwick chemical library (880 compounds) by FP-based competitive binding assay in order to isolate small specific molecules. Thus fourteen interacting compounds, already used on the market for specific pathologies, were found and then characterized by anisotropy titration ($IC_{50}<1\mu M$).
The selected molecules could be starting points to develop new therapeutic drugs acting on calmodulin.

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Keywords: Fluorescence polarization/anisotropy, FP-HTS, TRP-Calmodulin mutants, SYNCAM, fluorescent library.

NEW EVOLUTIONARY MODELS EXTEND THE STRUCTURE-FUNCTION PARADIGM FOR ANNEXINS

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We have studied the relationship between structure and function of annexins by computational sequence analysis of proteins and gene regulatory regions from over 2000 homologs. An updated phylogenetic tree describes the pattern and timing of gene duplications and the extent of evolutionary change in over 100 distinct subfamilies. Features of domain architecture are presented beside the evolutionary tree branches for all subfamilies to distinguish normal divergence from independent, convergent evolution of characteristics common to annexins in all eukaryotic kingdoms. Conservation analysis by hidden Markov models and divergence analysis were used to create unique molecular profiles for each protein subfamily and these were incorporated into 3D molecular models. The results permitted a visual assessment of the functionally important regions of each annexin protein and the molecular basis for divergent functions between subfamilies. Further detailed analysis revealed specific patterns of variation in surface exposed residues likely to influence membrane-binding kinetics and enable specific receptor interactions. These included a preponderance of bulky or hydrophobic residues (Trp, Phe, Tyr, Cys) in the exposed interhelical loops normally associated with carbonyl groups (Gly, Thr) and acidic residues (Glu, Asp) that form the canonical annexin calcium-binding domain. Many other subfamilies displayed a conserved pattern of basic residues, usually in the form of “K/R/H-G-D” motifs reminiscent of the ligand typical for transmembrane integrins. We interpret these data by proposing that the presence of either hydrophobic or basic residues in strategic, external sites normally occupied by calcium-coordinating residues reflects a spectrum of diverse mechanisms responsible for cell membrane interaction. This provides an original molecular basis for interpreting much experimental data that has long demonstrated differing calcium sensitivities, membrane phospholipid specificities, on-off kinetics and over all membrane affinity of different annexins. Key models of newly discovered annexins are presented to highlight these findings, including the first bacterial annexin, octad annexins unrelated to annexin A6, the expanded protist annexin family, the complete plant annexin family replete with defective calcium binding sites substituted by KGD motifs, and a multitude of unique subfamilies from invertebrates. The history of vertebrate annexins will be charted to trace their invertebrate origins and their rapid expansion in primitive fishes, the selective loss or amplification of individual subfamilies in specific lineages, and the emergence of human polymorphisms potentially associated with phenotypic differences.

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Keywords: Annexin, computational biology, evolution, functional determinants, membrane binding, receptor docking, structural motifs.

NEURONAL CALCIUM SENSOR PROTEINS: LOCALISATION IN HIPPOCAMPAL NEURONS AND DETERMINATION OF NOVEL BINDING PARTNERS.

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In neurons, variations in the cytosolic Ca^{2+} concentration regulate a plethora of different cellular events. The modulation of these neuronal calcium signals are achieved through the action of Ca^{2+} - sensor proteins, which in turn result in the transduction of these signals via binding with their own target proteins and membranes. The neuronal calcium sensor (NCS) proteins are members of the EF-hand containing super-family of Ca^{2+} binding proteins[1]. In certain NCS proteins, this first EF-hand is involved in the Ca^{2+}/myristoyl switch, in which binding of calcium results in a dramatic conformational change in the structure of the protein accompanied by the extrusion of the myristoyl tail. Ultimately this allows the protein to associate with both target proteins and membranes. Previous work in our lab has looked at the behaviour of these proteins in HeLa cells, were it was demonstrated that whilst NCS-1 did not show a Ca^{2+}/myristoyl switch[2], hippocalcin did[3]. Here I show that the previous observations of the localisation of the NCS proteins in cell lines also hold true for primary cultured rat Hippocampal neurons transfected to express fluorescently-tagged NCS proteins[4]. I also found that the physiological agonists glutamate/glycine, and the resulting rise in [Ca^{2+}], caused a significant and rapid translocation of hippocalcin-EYFP to distinct cellular structures including perinuclear Golgi-like structures within the Hippocampal neurons. The structural change of the NCS proteins upon binding calcium was also used to identify novel calcium dependent binding partners. Glutathione-S-transferase tagged variants of the NCS proteins hippocalcin and neurocalcin delta were used as bait in a large-scale pull down approach. Mass spectrometric analysis was then used to determine the identity of proteins that bound in a calcium dependent, and independent manner. I show here that CAPS and PDE are novel calcium dependent binding partners of hippocalcin and neurocalcin delta[5].

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Keywords: Ca^{2+} sensors, Cell signalling, NCS proteins, hippocampus, neuron

TYROSINE PHOSPHORYLATION OF ANNEXIN A2 REGULATES ACTIN MEDIATED CHANGES IN CELL MORPHOLOGY AND ADHESION

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Cell adhesion and motility require dynamic remodelling of the membrane-associated actin cytoskeleton in response to extracellular stimuli that are primarily transmitted through receptor tyrosine kinases. In search for mediators of the associated tyrosine phosphorylation signaling we here identify the membrane and actin-binding protein annexin A2 as a key regulator of insulin-induced actin cytoskeleton rearrangements. In BHK cells overexpressing the insulin receptor, generation of contractile force mediated by Rho/ROCK signaling caused dome-like accumulations of actin around the cell periphery and subsequent cellular detachment. These events correlated with an insulin-triggered tyrosine phosphorylation of annexin A2 and RNAi-mediated depletion of annexin A2 inhibited the insulin-induced morphological changes from occurring. In contrast, expression of a phospho-mimicking annexin A2 mutant, anx2-Y23E, was sufficient to drive peripheral actin accumulation and the resulting loss in cell adhesion in the absence of insulin stimulation. These results identify a tyrosine phosphorylation switch in annexin A2 as an important event in the regulation of actin-mediated changes in cell morphology and dynamics.

Keywords: annexin A2, Rho, actin binding protein, BHK cells, insulin, cell adhesion, phosphorylation

ANNEXIN A2 BINDS TO SPECIFIC RNA ELEMENTS IN THE 3'UTR OF ITS COGNATE mRNA

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Annexin A2 is a multifunctional Ca^{2+}-, F-actin and lipid-binding protein [1]. We have previously shown that a fraction of the Annexin A2 pool is recovered from mRNPs or polysomes associated with the cytoskeleton [2]. Specific proteins bind to specific sequences and/or structures in the 3'untranslated region (3'UTR) of mRNAs and are involved in their localisation in the cytoplasm. Thus, one of these proteins could be Annexin A2. The protein interacts *in vitro* and *in vivo* with c-*myc* and *annexin* A2 mRNAs in this subset of mRNPs, but not with β_2-*microglobulin* mRNA translated on membrane-bound polysomes. Annexin A2 binds to the localisation signal in the 3'UTR of c-*myc* mRNA [3]. A stem-loop structure within this localisation signal appears to be responsible for the targeting of c-*myc* mRNA to the perinuclear region [4]. Here we show that Annexin A2 binds to the first 150 nucleotides of the 3'UTR of *annexin* A2 mRNA, most likely involving a stem-loop structure. We are currently optimising the assay conditions to obtain crystals of a complex containing this region of the 3'UTR of *annexin* A2 mRNA bound to Annexin A2 protein. In collaboration with Robert H. Kretsinger (Charlottesville, USA), we have generated excellent crystals of Annexin A2 providing a resolution of 1.8 Å. The crystals of the Annexin A2-RNA complex diffracted to 3.4 Å resolution, however, each individual reflection was split or shattered, most likely reflecting cracking associated with freezing.

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Keywords: annexin A2, mRNP complex, mRNA, 3'UTR, crystal

CALCIUM-DEPENDENT BINDING OF S100A2 TO EZRIN *IN VITRO*

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S100A2 is a member of a multigene family of Ca^{2+}-modulated EF-hand type proteins thought to function within cells by binding to and thereby regulating in a Ca^{2+}-dependent manner. Some of the S100 proteins are located in the cell cortex and have been implicated in the regulation of cytoskeleton organization. Here we searched for specific S100A2 targets present in the cortical cytoskeleton and identified ezrin as such a specific target protein.
Ezrin bound by immobilized S100A2 in a Ca^{2+}-dependent manner. The binding requires the biologically active S100A2 since a F16A S100A2 mutant that does not form dimers is incapable of ezrin binding. By employing truncated ezrin derivative we also show that the interaction with S100A2 is mediated via the N-terminal domain of ezrin (N-ERMAD).
In order to identify specific amino acid residues involved in S100A2-ezrin complex formation we generated a series of C-terminal truncated S100A2 derivates and analyzed their interaction with N-ERMAD.
The impact of this novel interaction on ezrin membrane/ F-actin linking function and intracellulare localization will be discussed.

Keywords: ezrin, S100A2, cytoskeleton,F-actin

CITRULLINATED S100A3 BY PEPTIDYLARGININE DEIMINASES PROMOTED CALCIUM-DEPENDENT DIMERIZATION IN HUMAN HAIR FOLLICLE

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S100A3 is a unique member of the S100 protein family that exhibit the highest cysteine content and Zn^{2+} binding property, while its affinity to Ca^{2+} is very weak[1]. This protein is abundant in mature hair cuticles[2,3], and it is localized in the differentiating cuticular and cortical cells within the hair follicle[4]. Previous studies suggest close association of S100A3 with the epithelial differentiation leading to hair formation, but its molecular function in Ca^{2+}-signaling is still unknown. By 2D-PAGE-western blot analyses using a modified citrulline antibody, we found native S100A3 derived from the non-keratinized beard follicles. More than half of the arginine residues of S100A3 in the hair cuticles were found to be progressively converted to citrullines. Such conversion is known to be catalyzed by peptidylarginine deiminases (PAD; EC 3.5.3.15) in a Ca^{2+}-dependent manner[5]. Confocal immunofluorescent microscopy of the follicle tissues showed that the cytoplasmic S100A3 distribution within the differentiating cuticular cells is co-localized almost completely with PAD isozyme type III (PAD3) and partially with PAD2, but rarely with PAD1. Recombinant PAD1, PAD2 and PAD3 converted about 80%, 50% and 30% of ariginine residues in S100A3 in vitro. Native PAGE analyses showed that the enzymatic citrullination promoted the formation of S100A3 dimers, which are stable under lower Ca^{2+} concentration. Recombinant S100A3 modified by either PAD2 or PAD3 dimerized efficiently, despite the lesser conversion rates compared to that of PAD1. These results suggest that PAD enzymes modify arginine residues bound in S100A3. This modification increases the potential of Ca^{2+}-binding property during the hair epithelial differentiation.

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Keywords: citrulline, cuticle, hair follicle, peptidylarginine deiminase, S100A3

CRYSTAL STRUCTURES OF S100A2 IN ITS CALCIUM FREE AND LOADED STATE

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S100 proteins are involved in numerous physiological processes including cell cycle regulation, cell growth, differentiation and motility. Twenty members have been discovered in humans so far, and altogether, S100 proteins represent the largest subgroup within the EF-hand Ca^{2+}-protein family. Ca^{2+}-binding to S100 proteins triggers a large conformational change thereby translating the Ca^{2+} signal and initiating signal cascades (1).
Human S100A2 is unique among the S100 proteins because it is predominantly localized in the nucleus. S100A2 was identified as a tumour suppressor in human mammary epithelial cells, and downregulation of the protein was observed in tumour tissues of several cancer diseases. Recent studies show that S100A2 binds and activates p53 in a Ca^{2+}-dependent manner. This finding directly links the tumour suppressing activities of S100A2 and p53 and suggests a positive regulation of p53 through S100A2 (2).
In addition to Ca^{2+}, S100A2 binds Zn^{2+} with high affinity and abolishes Ca^{2+}-binding under physiological conditions, thereby rendering S100A2 inactive. To gain more insights into the structural basis of this regulation mechanism we crystallized S100A2 in the Ca^{2+}-loaded and in the Ca^{2+}-free form.
Crystals of Ca^{2+}-free S100A2 diffracted to 1.8 Å resolution. Phasing was performed by molecular replacement using the structure of metal-free S100A3 (3) as a search model. In the case of Ca^{2+}-loaded S100A2 a resolution of 1.4 Å was achieved.
The structure of Ca^{2+}-free S100A2 shows a compact dimer. Each subunit contains two EF-hands whereby the N-terminal EF-hands coordinate one Na^{+} ion in an octahedral manner. The helices of the C-terminal EF-hands adopt a closed antiparallel conformation. In the Ca^{2+}-bound form both EF-hands coordinate one Ca^{2+} ion in a pentagonal bipyramidal coordination sphere. Ca^{2+}-binding in the C-terminal EF-hands induce a large conformational change whereby helices H III of both subunits rotate about 90 degrees, similarly to S100B (4). This reorganization of the helical arrangement opens a large hydrophobic cavity in S100A2. The hydrophobic surface of this deep cavity represents the target binding-site for p53.

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Keywords: S100A2, p53, X-ray structure

STRUCTURAL BASIS OF THE INTERACTION BETWEEN S100B AND RAGE.

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S100 proteins are involved in intracellular and extracellular signal transduction. Like other Ca^{2+} sensor proteins S100 proteins undergo a conformational change upon Ca^{2+} binding which triggers target protein binding and regulation [1]. S100B acts extracellularly in the brain as neurite growth factor on neurons or as proinflammatory cytokine on glia cells. These extracellular signals are mediated by binding to the receptor for advanced glycation endproducts (RAGE) [2,3]. In order to study the interaction of S100B with RAGE in detail, we expressed and purified the different Ig domains of RAGE (V, C1 and C2 domain) in *E. coli*. The interaction of S100B with RAGE is strictly Ca^{2+} dependent and enforced by Zn^{2+} binding to S100B. We have shown that S100B binds specifically to RAGE V-domain by surface plasmon resonance and fluorescence titration studies. Interestingly, a recently described tetrameric form of S100B binds to RAGE with at least tenfold affinity compared to S100B dimer. The X-ray structure of tetrameric S100B reveals a close arrangement of the target binding sites on one face of the molecule [4]. In silico docking studies suggest that binding of tetrameric S100B can induce RAGE multimerisation, a prerequisite for signal transduction.
For structural analysis of RAGE we crystallized the major extracellular moiety of RAGE. The crystals diffract to 2.1 Å and the initial electron densities for model building are currently evaluated. The X-ray structure of RAGE will provide further insights into requirements and properties of extracellular S100 protein signaling.

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Keywords: S100B, RAGE, X-ray structure

ASSOCIATION OF CALPASTATIN WITH INACTIVE CALPAIN: A NOVEL MECHANISM TO CONTROL THE ACTIVATION OF THE PROTEASE?

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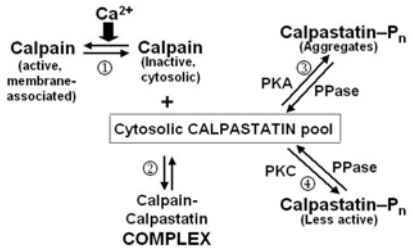
It is generally accepted that the Ca^{2+}-dependent interaction of calpain with calpastatin is the most relevant mechanism involved in the regulation of Ca^{2+} induced proteolysis. We now report that a calpain-calpastatin association can occur also in the absence of Ca^{2+} or at very low Ca^{2+} concentration, reflecting the physiological conditions in which calpain retains its inactive conformational state. The calpastatin binding region is localized in the non-inhibitory L-domain region containing the amino acid sequences coded by exons 4 to 7. This calpastatin region recognises a calpain sequence located near the end of the DII domain. Interaction of calpain with those calpastatins lacking these sequences becomes strictly Ca^{2+}-dependent since in these conditions the transition to an active state of the protease is an obligatory requirement. The occurrence of the molecular association between Ca^{2+} free calpain and various recombinant calpastatin forms has been demonstrated by the following experimental results. Addition of calpastatin protected calpain from trypsin digestion. Calpain co-precipitated when calpastatin was immunoprecipitated. The calpastatin molecular size increased following exposure to calpain. The two proteins co-migrated in zymogram analysis. Furthermore calpain-calpastatin interaction was perturbed by PKC phosphorylation occurring at sites located at the exons involved in the association. At a functional level, calpain-calpastatin interaction at physiological concentration of Ca^{2+} represents a novel mechanism for the control of the amount of the active form of the protease potentially generated in response to an intracellular Ca^{2+} influx.



Proposed model for the biochemical significance of the calpain-calpastatin interaction in physiological conditions. Following increase in intracellular free Ca^{2+}, the amount of calpain undergoing activation (①) is exclusively depending on the level of free calpain, which is maintained relatively low by interaction of the protease with calpastatin (②). The level of cytosol calpastatin is regulated by additional reactions (③ and ④), involving a PKA or PKC mediated phosphorylation.

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Keywords: Calcium, proteolysis, calpain, calpastatin, protein-protein interaction, enzyme regulation.

CLONING AND CHARACTERIZATION OF XENOPUS DICALCIN, AN S100-LIKE CALCIUM-BINDING PROTEIN IN XENOPUS EGG.

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Fertilization involves a species-specific fusion of sperm and egg, and a subsequent increase in the cytosolic calcium concentration in an egg, and resumption of its developmental program. To study the molecular mechanism of egg fertilization, we isolated a 26 kDa Ca^{2+}-binding protein from *Xenopus* eggs, a homologue of *Rana Catesbeiana* dicalcin (renamed from p26olf) that was isolated from the olfactory epithelium. The primary structure of *Xenopus* dicalcin shows 61% identity to that of *Rana* dicalcin and consists of two S100-like regions aligned in tandem, as seen in *Rana* dicalcin. Comared with other S100 members, C-terminal half of Xenopus dicalcin shares highest homology (~45%) to rabbit S100A11 and lesser homology (~38%) to rat S100A1. Genomic Southern blot analysis indicated that *Xenopus* dicalcin is a unique orthologue of *Rana* dicalcin. Recombinant *Xenopus* dicalcin can bind nearly four Ca^{2+} per molecule maximally. Northern blot analysis showed that *Xenopus* dicalcin is expressed in *Xenopus* eggs and also in other tissues. Immunohistochemical study revealed that *Xenopus* dicalcin is localized prominently in (i) the vitelline envelope and (ii) the cytoplasm of the cortex of both the animal and the vegetal hemispheres of *Xenopus* eggs. Several egg soluble proteins and two vitelline envelope proteins bound to *Xenopus* dicalcin in a Ca^{2+}-dependent manner under in vitro conditions, indicating the presence of target proteins of *Xenopus* dicalcin. A comparison of molecular masses of *Xenopus* dicalcin-binding proteins and known vitelline envelope proteins indicated that two of *Xenopus* dicalcin-binding proteins are gp37 and gp41. These results suggest that *Xenopus* dicalcin is a novel S100-like protein in eggs and is involved in the fertilization process.

Reference:
Miwa, N., and Kawamura, S. (2003) Frog p26olf, a molecule with two S100-like regions in a single peptide. *Microsc Res Tech* 60, 593-599

Keywords: Xenopus egg, fertilization, dicalcin, p26olf, S100

ANNEXINS AS INTRACELLULAR CALCIUM SENSORS

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The annexins are a multigene family of Ca^2- and charged phospholipid-binding proteins, which has been implicated in many Ca^{2+}-regulated processes inside the cell. Although diverse functions have been ascribed to annexins, there is no consensus about the role played by this family as a whole. We have compared the Ca^{2+}-induced changes in the localization of four members of the annexin family and of two truncated annexins in live cells, and demonstrated that these proteins have the ability to interact with the plasma membrane as well as with internal membrane systems in a highly coordinated, $[Ca^{2+}]_i$ -dependent manner. Annexin 2 was the most Ca^{2+} sensitive of the studied proteins, followed by annexin 6, annexin 4 and annexin 1. The calcium sensitivity of annexin 2 further increased following co-expression with S100A10 (p11). Upon elevation of $[Ca^{2+}]_i$, annexins 2 and 6 translocated to the plasma membrane, whereas annexins 4 and 1 also became associated with intracellular membranes and nuclear envelope. The NH_2-terminus had a modulatory effect on plasma membrane binding of annexins 1 and 2: its truncation increased the Ca^{2+} sensitivity of Anx 1, and decreased that of Anx 2. Given the fact that several annexins are present within any one cell, it is likely that they form a sophisticated $[Ca^{2+}]$ sensing system, with a regulatory influence on other signaling pathways.

Keywords: annexins, calcium, membrane binding

ENGINEERING OF mRNA CHIMAERAS TO STUDY THE ROLE OF ANNEXIN A2 IN mRNA TRANSPORT AND TRANSLATION

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We originally showed that Annexin A2 binds to mRNAs translated on cytoskeleton-bound polysomes (1) and is an integral component of specific mRNA-protein (mRNP) complexes (2). Furthermore, our results revealed that Annexin A2 binds to the localisation signal in the 3'-untranslated region (3'UTR) of c-*myc* mRNA (3). In general, the localisation signal for targeting resides in the 3'UTR of mRNAs. They are often difficult to define and appear to be recognised primarily at the level of RNA structure. Secondary RNA structures within *cis*-acting elements, in association with *trans*-acting proteins, have been implicated in the targeting of mRNAs. Annexin A2 may function as a *trans*-acting protein involved in the transport of specific mRNAs since it has been identified as a F-actin-binding (4), a nuclear shuttle protein (5) and a mRNA-binding protein (2, 3, 6), associated with a specific subpopulation of mRNPs (2). In order to study the role of Annexin A2 in mRNA localisation, we have generated several chimaeras consisting of a *Renilla Luciferase* reporter gene, hRLuc, attached to the cDNAs coding for the UTRs of c-*myc* and β_2-*microglobulin* mRNAs, in various combinations. The transcripts derived from each of these fragments have been tested for their binding to Annexin A2. Interestingly, GFP could not be used as a reporter since its mRNA associates with Annexin A2. The different chimaeras will be used for *in vitro* translation studies and subsequent *in vivo* mRNA localisation studies using real time spinning disc microscopy.

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Keywords: annexin A2, mRNP complex , mRNA, c-*myc*, Renilla Luciferase

S100A16 A NOVEL EF-HAND CALCIUM-BINDING PROTEIN BRAIN DISTRIBUTION AND Ca^{2+}-DEPENDENT NUCLEAR EXPORT

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S100A16 protein is a new and unique member of the EF-hand Ca^{2+}-binding proteins. S100 proteins are cell- and tissue-specific and are involved in many intra- and extracellular processes through interacting with specific target proteins. In the central nervous system (CNS) S100 proteins are implicated in cell proliferation, differentiation, migration, and apoptosis as well as in cognition. S100 proteins became of major interest because of their close association with brain pathologies like depression or Alzheimer disease. Here we report for the first time the purification and biochemical characterization of human and mouse recombinant S100A16 proteins. Flow dialysis revealed that both homodimeric S100A16 proteins bind two Ca^{2+} ions with the C-terminal EF-hand of each subunit, the human protein exhibiting a two fold higher affinity. Trp fluorescence variations indicate conformational changes in both orthologue proteins upon Ca^{2+}-binding, but formation of an hydrophobic patch, implicated in target protein recognition, only occurs in the human S100A16 protein. *In Situ* hybridization analysis and immunohistochemistry revealed a widespread distribution in the mouse brain. Furthermore, S100A16 expression was found to be astrocytes specific. Finally, we investigated S100A16 intracellular localization in human glioblastoma cells. The protein was found to be associated with nucleoli within nuclei and to translocate to the cytoplasm in response to Ca^{2+} stimulation.

Keywords: S100A16, EF-hand

EVALUATION OF S100A10, ANNEXIN II AND B-FABP EXPRESSION AS A MARKER FOR PATIENTS WITH RENAL CELL CARCINOMA

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This study aimed at an analysis of the expression of S100A10, annexin II and B-FABP genes in renal cell carcinoma (RCC) and their potential value as the tumor marker. Furthermore, any correlation between the gene expression and prognostic indicators of RCC was analysed.
Expression of each gene was estimated by RT-PCR in the non-neoplastic (normal) and tumor parts of resected kidney samples. Each antigen was immunostained in RCC and normal kidney tissues. Expression of S100A10 gene in average was 2.5 fold higher in the tumor than that in the normal tissues (n=47), after the standarization against that of β-actin. However, gene expression of annexin II, a natural ligand of S100A10, was only 1.64-fold higher in the tumor. In tissue sections of RCC, S100A10 and annexin II were membraneously immunostained. In the normal renal epithelia, however, both antigens were stained in the Bowman's capsule and the tubules from Henle's loop through collecting duct system but not in the proximal tubules, from where most RCCs are derived. On the other hand, expression of B-FABP gene was 20-fold higher in the tumor. No B-FABP was immunohistochemically detected in normal kidney sections, but it was cytoplasmically stained in RCC tissue sections. S100A10 and B-FABP genes were overexpressed in RCCs regardless of nuclear grade and stage. Immunopositivity in RCC tissues (n=13) was 100% for S100A10 and annexin II and 70% for B-FABP, but no clear relationship was observed in either antigen with nuclear grade and stage. It was concluded that the three were good as RCC markers. B-FABP was most specific to RCC, since it was little expressed in normal kidney tissues.

Keywords: S100A10, annexin II, B-FABP, renal cell carcinoma

PARASITE ANNEXIN B1 POSSESSES BIOCHEMICAL FEATURES SIMILAR TO MAMMALIAN AND PLANT ANNEXINS

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Annexin B1 from *Cysticercus cellulosae* has recently been identified by immunological screening in an attempt to find novel antigens for vaccine development against cysticercosis (1). The protein possesses anticoagulant activity and carries a significant therapeutic potential due to its thrombus-targeting and thrombolytic properties. We have investigated the biochemical properties of annexin B1 using liposome- and heparin-sepharose co-pelleting assays, as well as CD spectroscopy. The calcium-dependent binding to acidic phospholipid membranes is reminiscent of other mammalian annexins with a clear preference for high phosphatidylserine content (2). A unique property of annexin B1 is its ability to bind to liposomes with high phosphatidylserine content in the absence of calcium which might be due to the presence of several basic residues on the convex protein surface that harbours the membrane binding loops. Annexin B1 demonstrates lectin properties and binds to heparin sepharose in a cooperative, calcium-dependent manner. While this binding is reversible to a large extent, a small fraction of the protein remains bound to the glycosaminoglycan even in the presence of high concentrations of EDTA. Analogous to annexin A5 (3), we propose a model of heparin wrapped around the protein thereby engaging in calcium-dependent and –independent interactions. While the calcium-independent heparin binding sites identified in annexin A5 are not conserved, we hypothesise three possible sites in annexin B1. Results from CD spectroscopy and thermal denaturation indicate that, in solution, the protein binds calcium with a low affinity which leads to a slight increase in folding stability.

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Keywords: Annexins, heparin binding, membrane binding, parasite

IMPACT OF S-NITROSYLATION ON CATION BINDING AND CONFORMATION OF S100A1 AND S100B PROTEINS

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S-nitrosylation of cysteine thiols is an appreciated post-translational modification of proteins which seems to be as important as protein phosphorylation in signal transduction [1].

We have previously shown that S100A1 and S100B EF-hand Ca^{2+}-binding proteins possess highly reactive cysteine residues in the C-terminal parts of the proteins which are readily S-nitrosylated by an endogenous nitric oxide donor – S-nitrosoglutathione (GSNO) and that this modification influences conformation of the proteins [2]. We have also detected S100A1 and S100B among the S-nitrosylated proteins in bovine brain protein extract treated with GSNO.

In this work we present a more in-depth study of the S-nitrosylation-induced changes in S100A1 and S100B proteins. Using isothermal titration calorimetry (ITC) and chelator studies we show that S-nitrosylation modifies cation binding to S100A1 and S100B. Interestingly, detailed comparative NMR studies for apo S100A1 and S-nitrosylated apo S100A1 demonstrate that major conformational changes imposed by S-nitrosylation of a C-terminal cysteine are localized in the hinge region and the C-terminal helix H4 of S100A1 which are regions known to form the target binding sites for most S100 proteins.

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Keywords: S-nitrosylation, S100A1, S100B, zinc, calcium

Session 2 : Gene expression and calcium signaling

GENETIC DISSECTION OF THE FUNCTION OF RXRS AND THEIR HETERODIMERIC PARTNERS IN HOMEOSTASIS AND DISEASE OF ADULT MOUSE EPIDERMIS

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The functions of heterodimers formed between RXRs (alpha and beta) and other nuclear receptors (NRs ; e.g. RARs, PPARs, VDR, LXRs, FXR, …) in basal and suprabasal epidermal keratinocytes have been investigated in the adult mouse through a genetic approach using targeted spatio-temporally-controlled somatic mutagenesis (1), as well as through a pharmacological approach using topical treatments of the skin with NR-specific agonists and antagonists.

Our results (2-6) concerning the role of NRs in the molecular and cellular events that underlie the homeostatic renewal of the epidermis and the involvement of keratinocytes in skin disease (atopic dermatitis), will be presented and discussed.

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Keywords: nuclear receptors, kertinocytes, epidermis, nuclear calcium

CALCIUM TRANSIENTS AND CALCIUM SIGNALING DURING EARLY NEUROGENESIS IN THE AMPHIBIAN EMBRYO XENOPUS LAEVIS

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Development of the vertebrate embryonic nervous system is characterized by a cascade of signaling events. In *Xenopus*, the initial step in this cascade results from signals emanating from the dorsal mesoderm that divert the fate of the ectoderm from an epidermal to a neural lineage. These signals include extracellular antagonists of the bone morphogenetic protein (BMPs). Experiments performed with isolated ectoderm suggest that epidermis is induced by BMP, whereas neural fates arise by default following BMP inhibition; however we show that this mechanism is not sufficient for neural determination.

Ca^{2+} imaging of intact *Xenopus* embryos reveals patterns of Ca^{2+} transients in the dorsal ectoderm but not in the ventral ectoderm [1]. These increases in internal calcium concentration ($[Ca^{2+}]_i$) occurring via the activation of dihydropyridines (DHP) calcium channels are necessary and sufficient to orientate the ectodermal cells toward neural fate [2,3]. In one hand the treatments which antagonize the increase in $[Ca^{2+}]_i$ inhibits the neuralization. In other hand, the artificial increase in $[Ca^{2+}]_i$ whatever its origin neuralize the ectoderm. Using these properties we have constructed a subtractive cDNA library between untreated ectoderms and caffeine-treated ectoderms to increase $[Ca^{2+}]_i$ and orientate the cells towards the neural pathway. We have identified early Ca^{2+} target genes expressed in neural territories. One of these genes, an arginine methyl transferase, controls the expression of the early proneural gene*, Zic3*, [4].

Here we discuss an alternative model where calcium plays a central regulatory role in early neurogenesis. This model integrates the activation of a Ca^{2+}-dependent signaling pathway due to an influx of Ca^{2+} through dihydropyridine Ca^{2+} channels. While Ca^{2+} is required for neural determination, epidermal determination occurs when Ca^{2+} -dependent signaling pathways are inactive.

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Keywords: calcium, DHP-channels, neural determination, Xenopus laevis, gene expression

CYTOSOLIC AND NUCLEAR CALCIUM SIGNALING IN PLANT CELLS: REGULATION AND BIOLOGICAL IMPLICATIONS

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Plants respond to abiotic and biotic signals by specific changes in cytosolic and nuclear calcium concentrations. Here, we show that these changes involve cross-talk with other second messengers and impact on plant cell metabolism and viability. We will present qualitative and quantitative data linking sequential steps of a signaling pathway starting from the initial recognition of a signal by plasma membrane-bound receptors to end up with the accumulation of aromatic compounds. Identification of potential regulatory processes will be discussed.

Keywords: nuclear calcium, plant cell

ANALYSES OF CALCIUM-DEPENDENT INTERACTIONS BETWEEN ALG-2 AND ITS BINDING PARTNERS INVOLVED IN MEMBRANE TRAFFICKING

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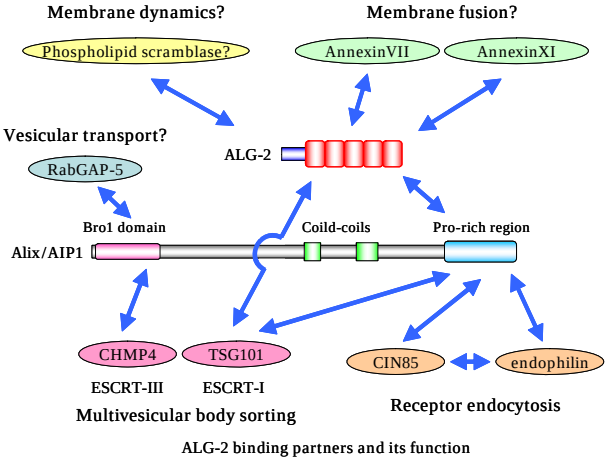
ALG-2 (apoptosis linked gene-2) is a member of penta-EF-hand Ca^{2+}-binding proteins and interacts with target proteins, such as Alix, TSG101, Annexin VII and Annexin XI (1). Previously, we reported that ALG-2 recognizes four tandem PxY repeats in Pro-rich region (PRR) of Alix (2). To search for new ALG-2 binding proteins, BLAST search was performed using the ALG-2 binding sequence in Alix. Among several candidates, we picked up phospholipid scramblase 3 (PLSCR3), which has PRR containing PxY sequence in its N-terminal region. We analyzed binding properties of ALG-2 with PLSCR3. By pull-down assay using ALG-2 fused with glutathione-*S*-transferase (GST-ALG-2) and ALG-2 fused with green fluorescent protein (GFP-PLSCR3) from HEK293 cells, ALG-2 bound specifically to PLSCR3 Ca^{2+}-dependently. Analysis of PLSCR3 deletion mutants revealed that PLSCR3 has two ALG-2 binding sites, ABS-1 containing PxY and a new binding site, ABS-2 in its PRR. An ALG-2 splicing variant lacking G121 and F122 (designated ALG-2ΔGF) associated with PLSCR3, but not with Alix. ALG-2ΔGF associated with ABS-1 deletion mutant of PLSCR3 but not with ABS-2 deletion mutant. Different residues in ALG-2 was found to be important for binding to each ABS in PLSCR3 by analysis of various ALG-2 amino acid substitution mutants. These results suggest that ALG-2 interacts with target sequences by using different interfaces.



ALG-2 binding partners and its function

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Keywords: apoptosis-linked gene-2, penta-EF-hand, Pro-rich region, protein-protein interaction, surface plasmon resonance

ROLE OF DREAM, A TRANSCRIPTIONNAL REPRESSOR REGULATED BY CALCIUM, IN IMMUNE SYSTEM

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The transcriptional repressor Downstream Regulatory Element Antagonist Modulator (DREAM) is a Ca^{2+}-binding protein that binds specifically to DRE sequences in DNA (1). Binding of DREAM to DREs is regulated by the level of nuclear Ca^{2+}, by the interaction with other nucleoproteins like aCREM (2) and CREB (3) and by the PI3 kinase pathway (4). Mutation of two key amino acids within any of the functional EF hands of DREAM (EFmDREAM) results in a protein insensitive to Ca^{2+} that remains bound to DNA during Ca^{2+} stimulation. Since DREAM binds to DRE sites as a tetramer, EFmDREAM was proposed to function as a dominant active mutant in a background of wild-type (wt) DREAM. DREAM is highly expressed in many brain areas and in some peripheral organs, including thyroid gland, testis and the thymus. To understand the functional significance of DREAM in the immune system, we have prepared transgenic mice expressing dominant active mutant of DREAM (EFmDREAM). Transgenic T-cells exhibited decreased interleukin (IL)-2, -4 and interferon $(IFN)_{\gamma}$ production after polyclonal activation and following antigen-specific response. Chromatin immunoprecipitation and transfection assays showed that DREAM binds to and represses transcription from these cytokine promoters (5). These data propose DREAM as a Ca^{2+}-dependent repressor of the T cells response. Actually, we are studying the role of DREAM on the function of B lymphocytes, particurlarly in the production of immunoglubulines.

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Keywords: Calcium, immune system, transcription, Ca^{2+}-binding protein

THE GUARD CELL CALCIUM - BASED SIGNALING NETWORK

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Stomata are pores found on the surfaces of plant leaves. They control the uptake of carbon dioxide for photosynthesis and the loss of water vapour during the process of transpiration. The aperture of the stomatal pore is governed by the state of turgor of the two guard cells that surround the stomatal pore. When the guard cells are fully turgid the pore gapes open allowing gas exchange and conversely stomatal closure is associated with a loss of turgor. A wide range of environmental signals and plant hormones contribute to the control of stomatal aperture. Underlying changes in guard cell turgor and hence stomatal movements is a complex intracellular signaling network. An increase in the concentration of guard cell cytosolic free calcium ions has been shown to be involved in the response to many different signals. This lecture will discuss evidence that guard cell ABA calcium – based signaling is organised on a network basis and consider recent data relating to S1P signaling in guard cells.

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Keywords: stomata, calcium, guard cell, signaling network, plant cell

Ca^{2+} SIGNALING DURING EARLY EMBRYONIC PATTERNING IN ZEBRAFISH

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It has been proposed that Ca^{2+} signaling, in the form of pulses, waves and steady gradients, may play a crucial role in key pattern forming events during early vertebrate development[1-3]. Using the zebrafish (*Danio rerio*) as a highly applicable system that lends itself particularly well to imaging studies, aequorin-based imaging data will be presented to illustrate the various forms of Ca^{2+} transients that accompany development from the late Blastula, through Gastrula, and into the early Segmentation Period. This developmental window contains the major pattern forming movements of epiboly, involution, and convergent extension, which result in the establishment of the basic germ layers and body axes[4]. Data will be presented to support the suggestion that propagating waves (both long and short range) of Ca^{2+} release, followed by sequestration, may play a crucial role in coordinating cell movements during these pattern forming events, as well as helping to define the morphological boundaries of specific embryonic structures and organ anlagen[5,6]. The various potential targets of these Ca^{2+} transients will also be discussed as well as how they might integrate with other identified pattern forming pathways known to modulate early developmental events (such as the Wnt/Ca^{2+} pathway[7]). Work supported by Hong Kong Research Grants Council grants: HKUST6016/01M, HKUST6214/02M, HKUST6279/03M and HKUST6241/04M.

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Keywords: Ca^{2+}, pattern formation, zebrafish, gastrulation

MOLECULAR MECHANISMS UNDERLING X-LINKED MYOTUBULAR MYOPATHY

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X-linked myotubular myopathy (XLMTM) is a severe congenital muscular disorder due to mutations in the *MTM1* gene, which encodes a lipid phosphatase named myotubularin. Myotubularin acts on phosphatidylinositol 3- and 3,5-phosphate (PtdIns3P and PtdIns3,5P*2*), second messengers that regulates the endocytic pathway. In order to understand the molecular mechanisms underlying the pathogenesis of the disease, we have generated constitutive and muscle-specific myotubularin deficient mouse lines. *Mtm1* knockout mice develop a progressive generalized myopathy with reduced life expectancy, and present a muscle pathology that resembles that of XLMTM patients. Furthermore, tissue-specific inactivation of the *Mtm1* gene has revealed that skeletal muscle is the primary tissue implicated in the disease.

The aim of our study is to identify the molecular cascades leading to XLMTM pathogenesis. For this, we are analysing the transcriptome of myotubularin deficient mouse skeletal muscle before and after the appearance of pathological signs (2 and 6 weeks respectively). The first group (2 weeks) shows no statistical differences between WT and KO muscles whereas the second group (6 weeks) shows a highly active transcriptional response to myotubularin deficiency in skeletal muscle that involves genes related to vesicle trafficking, ion homeostasis, transcription, protein metabolism and muscle development.

Among these pathways, we are planning to analyse whether the Ca^{2+} homeotasis involved in the excitation-contraction coupling machinery is altered in *Mtm1*-deficient skeletal muscle. If this is the case, a deregulation in handling the intracellular calcium concentration during contraction/relaxation should have profound consequences in muscle physiology.

Using Q-PCR we have confirmed the transcriptional deregulation of many genes implicated in Ca^{2+} homeostasis in skeletal muscles of 6 week-old mice. Interestingly, we have found that the expression of several genes involved in this pathway is altered in early pathological phases (2 weeks). In particular, we have identified two genes, Sln and rRad, as early markers of the murine myotubular myopathy. We are currently analysing these transcriptional deregulations at post-transcriptional level. Depending on the results, fonctional studies on calcium-dependent pathways will be undertaken in myotubularin deficient muscle

Keywords: Myopathy, myotubularin, endocytic pathway,transcriptoma, cells, Ca homeostasis.

S100B IN MYOBLASTS INHIBITS MYOTUBE FORMATION BY INTERFERING WITH MYOD EXPRESSION

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Besides being expressed in high abundance in astrocytes (1), S100B is expressed in relatively large amounts in several non-nervous cell types including myoblasts, the precursors of skeletal muscle fibers (2). S100B has been shown to participate in the regulation of several intracellular processes including cell cycle progression and differentiation. For example, S100B inactivates and reduces the expression of the tumor suppressor protein, p53, in melanoma cells (3), thereby probably favoring melanoma cell proliferation, and stimulates PC12 cell proliferation and inhibits NGF-induced PC12 cell differentiation via activation of an Akt-$p21^{WAF1}$-cdk4-Rb pathway (4). We sought to investigate regulatory effects of S100B within myoblasts by two distinct experimental approaches, i.e. stable overexpression of S100B and inhibition of S100B expression by RNA interference. Overexpression of S100B in myoblasts (clone L6C8) resulted in inhibition of myoblast fusion into myotubes, suggesting that intracellular S100B might interfere with myoblast differentiation. Indeed, little or no expression of the muscle-specific transcription factor, myogenin, and the late differentiation marker, myosin heavy chain (MHC), were detected in L6C8 myoblasts compared with mock-transfected myoblasts (clone L6C11). However, these effects were not consequent to increased proliferation of L6C8 myoblasts. Interestingly, no obvious decrease in the extent of activation of p38 MAPK, which is crucial for myoblast terminal differentiation, could be seen in L6C8 myoblasts under differentiation conditions. Also, transfection with MKK6EE, an active mutant of the p38 MAPK upstream kinase MKK6, while stimulating the extent of myogenin expression in L6C11 myoblasts as expected, failed to counteract the inhibitory effect of S100B overexpression on myogenin expression in L6C8 myoblasts. Thus, S100B might interfere with myoblast differentiation and myotube formation by acting downstream of p38 MAPK. Inability of L6C8 myoblast to form myotubes was accompanied by reduced $p21^{WAF1}$ induction and MyoD expression while Myf-5 expression was unchanged, compared with L6C11 myoblasts. Also, transient transfection with the muscle-specific transcription factor, MyoD, but not Myf-5, restored L6C8 myoblast differentiation and fusion in part, while silencing S100B expression in wild-type myoblasts by RNA interference resulted in stimulation of MyoD, myogenin and MHC expression and myotube formation. Also, the transcriptional activity of NF-κB, a negative regulator of MyoD expression (5), was greatly enhanced in L6C8 myoblasts. The present data support the possibility that intracellular S100B might take part in the regulation myoblast differentiation by interfering with MyoD expression in an NF-κB-dependent manner.

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Keywords: S100B, myoblast, proliferation, differentiation, modulation, quiescence.

COMPLEX REGULATORY EFFECTS OF EXTRACELLULAR S100B ON MYOBLAST DIFFERENTIATION: S100B ACTIVATES QUIESCENT MYOBLASTS AND SATELLITE CELLS

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We reported that administration of pM amounts of S100B to high-density myoblast cell lines or primary myoblasts (i.e., satellite cells) in differentiation medium (DM) results in inhibition of differentiation, stimulation of proliferation and inhibition of apoptosis via inhibition of the MKK6-p38 MAPK pathway, stimulation of a Ras-MEK-ERK1/2-cyclin D1-Rb pathway, and stimulation of a Ras-MEK-ERK1/2-NF-κB pathway, respectively (1,2). Thus, S100B might participate in embryonic myogenesis and muscle regeneration by stimulating myoblast/satellite cell proliferation thereby contributing to the attainment of the critical cell density required for the subsequent myoblast fusion into myotubes and/or the damaged skeletal muscle fibers, and by modulating myotube and/or myofiber hypertrophy. To validate this hypothesis, we analyzed effects of a single administration of pM amounts of S100B to low-density myoblasts in DM. Exposure of myoblasts in DM to 100 pM S100B for 24 h only resulted in stimulation of myotube formation (as measured 3 days after withdrawal of S100B); a second administration of S100B at day 3 (for 24 h) resulted in myotube hypertrophy; and administration of S100B on day 3 only (for 24 h) also resulted in myotube hypertrophy. Because non-fused myoblasts co-existing with myotubes at day 3 are quiescent, these results suggest that S100B might activate quiescent (G_0-arrested) myoblasts and stimulate their proliferation, thereby accelerating myotube formation/hypertrophy. To support this possibility, we made myoblasts quiescent, switched them to DMEM for 16 h and analyzed them for cell cycle by FACS. S100B decreased the fraction of myoblasts in G_0/G_1 phase and increased the fraction of myoblasts in S phase, indicating that S100B had activated quiescent myoblasts and stimulated their proliferation. These events were accompanied by a rapid (within minutes) activation of ERK1/2, p38 MAPK and Akt, and in a typical time-course of changes in the levels of the muscle-specific transcription factors Myf-5 and MyoD (3). Also, S100B stimulated myoblast and satellite cell migration. However, the overall effect of S100B on myoblast differentiation was strongly dependent on the time of exposure and myoblast density: a long exposure (i.e., 3-4 days) to S100B resulted in inhibition of differentiation irrespective of myoblast density as did a brief (24 h) exposure of myoblasts at high density (with stimulation of ERK1/2 and inhibition of p38 MAPK), as opposed to stimulation of differentiation in the case of low-density myoblasts (which showed activated ERK1/2 and p38 MAPK). Thus, both the extent of cell-cell contacts and the exposure time appear to condition the sensitivity of myoblasts to S100B, suggesting that S100B might play a dual regulatory role during embryonic myogenesis and muscle regeneration.

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Keywords: S100B, myoblast, satellite cell, quiescence, proliferation, differentiation.

THE CALCIUM-BINDING PROTEIN ALG-2 INTERACTS WITH THE RNA-BINDING PROTEIN AIP-2, A POTENTIAL REGULATOR OF ALTERNATIVE SPLICING AND CELL DIVISION

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Ca^{2+} and calcium binding proteins are one of the key regulators of many cellular processes. ALG-2 is a highly conserved calcium binding protein belonging to a family of penta EF-hand proteins. ALG-2 which originally has been linked to apoptotic pathways (1), is significantly upregulated in highly proliferating cells including cancerous tissues of a number of different tumors (2,3). We used yeast two hybrid approach to identify targets which may interact with ALG-2. One of the proteins identified in this screen was a highly conserved RNA-binding protein which we named AIP2 for **A**LG **i**nteracting **p**rotein **2**. Later it was found that homologues of AIP2 may be essential for the regulation of RNA alternative splicing (4) and cell division (5). In this study we further characterize the interaction between ALG2 and AIP2. NIH 3T3 cells were transfected with expression vectors encoding either ALG2-mRFP or AIP2-GFP, or both, followed by confocal microscopy. In cells expressing ALG2-mRFP only, the protein was predominantly located in the cytoplasm whereas AIP2-GFP alone was located in the nucleus. In sharp contrast, when NIH 3T3 cells expressed both proteins a significant amount of ALG2-mRFP co-localized with AIP2-GFP within the nucleus. In order to test whether nuclear co-localization of the two proteins could also be observed *in vivo* we injected similar fluorescent constructs into zebrafish embryos at the 1-cell stage and observed a similar cellular distribution of the 2 proteins. Especially striking was the nuclear co-localization of the two proteins during development if we co-injected the 2 constructs at the 1-cell stage. Formation of complexes between ALG2 and AIP2 may thus play an important role in calcium-dependent signaling influencing alternative splicing and cell division during development.

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Keywords: Penta EF-protein; ALG-2; AIP-2; confocal microscopy; zebrafish; alternative splicing

THE ROLE OF CALCIUM IONS AND FGF SIGNALING DURING NEURAL INDUCTION IN XENOPUS

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In amphibians, neural induction takes place on the dorsal face of the embryo and involves a specific tissue interaction between the dorsal ectoderm and the involuting dorsal mesoderm. In the absence of this interaction, the ectoderm will give rise to epidermis. The traditional dogma suggests that neural induction occurs via a default process, resulting from the interaction of antagonising dorsal signals (via members of the TGFβ family, i.e., BMP4 and 7) that control epidermal determination, and dorsalizing signals secreted from the dorsal mesoderm, such as noggin, chordin and follistatin. Dorsalizing molecules allow the neural fate of the dorsal ectoderm to be revealed by inhibiting the binding of BMPs on their receptors.However, it has been shown that an increase in intracellular calcium in the dorsal ectoderm is both necessary and sufficient signal to reorientate the ectoderm cell fate from epidermis towards a neural pathway (Leclerc *et al*. 2000). Under these conditions, neural induction does not occur by default process but is instead considered to be an instructive process. Calcium increase occurs as transient flashes in distinct groups of cells with the calcium entering the cells through L-type calcium channels (Moreau and Leclerc. 2004, Leclerc *et al*. 2000). Calcium has been shown to control the expression of at least two early transcription factors, *XlPOU2* and *Zic3* that are specific for neural determination. Recent evidence indicates that FGF signaling play an important role in neural induction in *Xenopus* (Delaune *et al*. 2005, Stern, 2005). However, the relationship between the calcium increase and the FGF signaling is as yet unknown. Here, we show that FGF (100 ng/mL) induces an elevation in calcium concentration in animal caps preloaded with aequorin. This calcium increase is inhibited when the animal cap is pretreated with nifedipine, an antagonist of dihydropyridine-sensitive calcium channels. In addition, RT-PCR studies show that the expression of *Zic3* is reduced in animal caps treated with SU5402, a FGFR inhibitor. We propose that activation of FGF receptors triggers a calcium entry via TRP and/or L-type calcium channels. The opening of calcium channels is probably due to production of arachidonic acid and eicosanoids.

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Keywords: Calcium, neural induction, FGF, aequorin, *Xenopus*, *Zic3*

ISOLATED PLANT NUCLEI GENERATE CALCIUM CHANGES IN RESPONSE TO EXTERNAL STIMULI

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The calcium ion (Ca^{2+}) is firmly established as a ubiquitous intracellular second messenger mediating responses to a variety of biotic/abiotic stress signals in plants. Here, we show that nuclei isolated from cell suspension cultures of BY-2 tobacco, harbouring the bioluminescent Ca^{2+} probe aequorin in the nucleoplasm, convert physical stimuli into changes in free Ca^{2+} concentrations in the nucleoplasm. The system implicates the coordination of the buffering capacity of the nucleoplasm and the activities of calcium channel/pump. A combined approach including a mathematical modeling and use of pharmacologically active compounds suggests that these channels and pumps localize to the inner membrane of the nuclear envelope. Our data suggest further that Transient Potential Receptor-*like* channel induce nuclear Ca^{2+} variation both in intact cell and in isolated nuclei. Collectively, our data strengthen the fact that nucleus is partially autonomous in terms of calcium signaling and may control downstream nuclear Ca^{2+}-dependant events.

Keywords: nuclear calcium, tobacco BY-2 cell line, aequorin, mathematical modeling, mechanical stress

A SUBTRACTIVE APPROACH FOR CALCIUM-INDUCED GENES INVOLVED IN XENOPUS NEURAL FATE

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In the early *Xenopus* gastrula, ectodermal cells can adopt either an epidermal or a neural pathway. The neural fate is conferred during gastrulation by neuralizing factors such as noggin, chordin and follistatine secreted by the dorsal mesoderm. These factors act by sequestering Bone Morphogenetic Protein-4 (BMP-4) responsible of the epidermal fate. However, in amphibian embryo, we have previously shown that calcium (Ca^{2+}) plays an active role in neural induction: spontaneous Ca^{2+} transients, occurring via the activation of L-type voltage-sensitive Ca^{2+} channels (LTC), are recorded in the dorsal ectoderm during gastrulation (Leclerc et al. 2000). The blockade internal calcium concentration ($[Ca^{2+}]_i$) increase either by LTC antagonists or Ca^{2+} chelator inhibits neural induction. In addition increase in $[Ca^{2+}]_i$ either by activation of LTC or caffeine treatment which induces a release of Ca^{2+} from internal stores, trigger neural induction. Thus an increase in Ca^{2+} is both necessary and sufficient to commit ectoderm cells to the neural pathway (Moreau & Leclerc 2004). The accumulated Ca^{2+} pattern is correlated with the expression of the early proneural gene *Zic3* (Leclerc et al, 2003). However, the molecular mechanism by which Ca^{2+} orientates the cells towards a neural fate remains elusive.

To identify Ca^{2+} target genes involved in neural induction, we constructed a subtractive cDNA library between untreated (i.e. ectodermal) and caffeine-treated (i.e. neuralized) isolated ectoderms to release Ca^{2+} (Batut et al. 2003).

The calcium-triggered genes exhibit expression patterns specifically restricted to the neural territories during development. We describe the characterisation of one of the Ca^{2+} target genes, xPRMT1b (**X**enopus **P**rotein a**R**ginine **M**ethyl**T**ransferase type I b), which encodes an arginine N-methyltransferase. We analyzed the functional role of *xPRMT1b*, that it is a direct Ca^{2+} target gene involved in the control of neural gene expression. Its invalidation by microinjection specific antisense morpholino impairs the development of anterior neural structures (brain and eyes) (Batut et al, 2005).

Our results indicate that *xPRMT1b* mediates Ca^{2+} signaling by playing a key role in the control of the epidermal versus neural cell fate decision. We next would like to investigate the regulations by which calcium variation activates proneural gene transcription.

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Keywords: Xenopus embryo, neural induction, calcium, gene expression, arginine N methyl transferase

ANTI-APOPTOTIC FUNCTIONS OF ALG-2 IN HUMAN CANCER CELLS

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A highly controlled balance between proliferation and apoptotic processes exists in all higher eukaryotic organisms. Failure in regulation of these processes may lead to disturbed cell growth and thereby possibly to the development of cancer. Molecules involved in these processes are therefore prime candidates to be used as diagnostic or prognostic markers and may also be considered as potential therapeutic targets. The calcium-binding protein ALG-2 (Apoptosis-Linked Gene-2 encoded protein) was initially identified as a factor needed for T-cell receptor mediated apoptosis (1). However, this pro-apoptotic function of ALG-2 has not been confirmed in human cells. Work in our laboratory has shown that ALG-2 is highly expressed in several different cancers (2) indicating that ALG-2 may permit high viability and/or increased proliferation. Therefore, down-regulation of ALG-2 might be a possible strategy for anti-proliferative therapy. Efficient down regulation of ALG-2 has been obtained in HeLa and U2OS cells using two different siRNA molecules. Down regulation of ALG-2 led to accumulation of cells in the G2/M phase, reduced the proliferation rate, increased the population of non-viable cells and led to a higher sensitivity towards death inducing agents. These results show that ALG-2 functions as an anti-apoptotic protein in our experimental set up, and not as a pro-apoptotic protein as previously published by others (1).

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Keywords: ALG-2, Calcium signaling, Apoptosis, Cell cycle, siRNA

EPIGENETIC REGULATION OF S100A6 (CALCYCLIN) GENE EXPRESSION

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S100A6 (calcyclin) is a calcium binding protein of the S100 family. Members of this protein family are often expressed in a cell-specific manner. S100A6 is especially abundant in fibroblasts and epithelial cells (1), is present in some neurons and astrocytes (2) but is absent in various other cell types. We have shown earlier that S100A6 expression may be reactivated in cells treated with 5-azacytidine (3), an inhibitor of DNA methyltransferase, indicating that DNA methylation may block S100A6 transcription. In this study we have explored differences in the epigenetic marks i.e., histone modifications and DNA methylation within the S100A6 gene promoter in cells with active S100A6 expression (Hep-2) and those that do not express S100A6 (HEK293). Using chromatin immunoprecipitation (ChIP) we show that the level of histone H3 acetylation is much higher in Hep-2 than in HEK293 cells. On the contrary, methylation of Lys 9 and Lys 27 in histone H3, considered to be characteristic for an inactive chromatin, prevails in the S100A6 gene promoter in HEK293 cells. Bisulfite modification analysis also showed differences in cytosine methylation within the S100A6 gene between Hep-2 and HEK293 cells. However, in spite of the differences in epigenetic marks and hence in chromatin organisation within the S100A6 gene promoter, the USF transcription factor, essential for S100A6 gene promoter activity (4,5), can bind to the promoter in both cell types. Thus we show that the expression of S100A6 is controlled by an epigenetic mechanism.

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Keywords: S100A6, calcyclin, promoter, USF, chromatin immunoprecipitation, DNA methylation

Session 3 : Calcium signaling and trafficking

PHOSPHOLIPASE D1 IN CALCIUM-REGULATED EXOCYTOSIS: A CHECKPOINT FOR MONOMERIC GTPASES

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Phospholipase D (PLD) generates phosphatidic acid (PA), a multifunctional lipid that can activate selected enzymes, serve as protein attachment site or alter membrane curvature and facilitate fusion. In neuroendocrine chromaffin and PC12 cells, PLD1 is present at the plasma membrane. We previously described that PLD1 is activated in response to secretagogue-evoked stimulation and its activation correlates in timing and Ca^{2+}-dependence with exocytosis. Moreover, inhibitors of PLD-derived PA production as well as expression or injection of catalytically inactive PLD1 mutants inhibit secretion, suggesting that PLD1 might play an important function in the exocytotic machinery[1]. To further probe this idea, we investigated the intracellular distribution of PA in fixed and living cells. Using a fluorescent PA-binding probe, we found that PA appears at the sites of active exocytosis. siRNA experiments to reduce endogenous PLD1 level confirm the functional importance of PLD1-derived PA in large dense-core granule exocytosis. Exocytosis can be restored in PLD1-deprived cells by the exogenous addition of inverted-cone shaped lipids, suggesting that the role that PA fulfils in exocytosis might be related to its effect on lipid bi-layer curvature at the granule docking sites, thereby facilitating fusion. Accordingly, microinjection of a catalytically-inactive PLD1 mutant in chromaffin cells increases the rise time of the single exocytotic spikes resolved by amperometry, in line with an alteration of the fusion and/or pore expansion events.

The possible implication of PLD1 in the late exocytotic fusion process implies a tight temporal regulation of its enzymatic activity. In chromaffin and PC12 cells, PLD1 is activated by the granule associated ARF6 GTPase after secretagogue-induced recruitment and docking of granules onto the plasma membrane[2]. We recently found that RalA, another GTPase that potentially regulates PLD1 activity, is also activated at the plasma membrane during exocytosis[3]. Expression of a constitutively active RalA mutant enhances secretion from PC12 cells. Conversely, expression of an inactive GDP-bound RalA protein or silencing of the RalA gene by RNA interference leads to a strong impairment of the exocytotic response. Cell stimulation triggers a direct interaction between RalA and ARF6-activated PLD1 at the plasma membrane. Moreover, reduction of endogenous RalA expression level prevents the activation of PLD1 observed in secretagogue-stimulated cells. We propose that RalA together with ARF6 participate in the signaling pathways that integrate calcium signals to PLD1 and its lipid-modifying activity at the sites of exocytosis.

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Keywords: Exocytosis, phospholipase D, membrane fusion, secretory granules, neuroendocrine cells

Ca^{2+}-DEPENDENT REGULATION OF SYNAPTIC VESICLE PRIMING AND SYNAPTIC SHORT-TERM PLASTICITY

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The efficacy of synaptic transmission between neurons can be altered transiently during neuronal network activity. This phenomenon of short-term plasticity is a key determinant of neuronal network properties and is involved in many physiological processes such as motor control, sound localization, or sensory adaptation. Synaptic short-term plasticity is critically dependent on cytosolic [Ca^{2+}], which in turn activates essential synaptic vesicle priming proteins of the Munc13-family in a calmodulin-dependent and diacylglycerol-dependent manner (Rhee et al., 2002; Junge et al., 2004).

In my seminar, I will discuss studies concerned with the characterization of Ca^{2+}/calmodulin binding to Munc13s, and with the regulation of Munc13 function and Ca^{2+}-sensitivity by protein components of the presynaptic active zone. Our data show that convergent evolution created structurally unrelated high-affinity Ca^{2+}/calmodulin binding sites in all known Munc13 isoforms, which regulate the respective Munc13 priming activity. A similar convergent evolutionary process generated structurally unrelated binding sites for different active zone scaffolding proteins in the different known Munc13 isoforms, which are critical determinants of Munc13 trafficking to synapses and of their priming function.

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Keywords: synaptic vesicles, plasticity, calmodulin binding, Mun13, trafficking.

THE SPCA-TYPE OF Ca^{2+}-TRANSPORT ATPASES

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Ca^{2+} in the lumen of intracellular compartments like the endoplasmic reticulum (ER), the Golgi apparatus (GA) and the more distal compartments of the secretory pathway is indispensable for a number of processing steps in the production of membrane and secretory proteins. These processing steps include mainly glycosylation and endoproteolytic cleavage. At the same time luminal Ca^{2+} represents a store of activator Ca^{2+} that can be released to control many cytosolic processes. The relative amounts of Ca^{2+} stored in the ER and the GA vary with the cell type, but the ER represents the functionally most important IP_3-releasable Ca^{2+} store controlling cytosolic signaling. Although in animal cells SERCA and the ER occupy a central position in Ca^{2+} signaling, the P-type SPCA (secretory pathway Ca^{2+} ATPase) pumps found in the GA may be evolutionary older. Most animal cells express SPCA1 from the *ATP2C1* gene (human nomenclature). Four different splice variants (SPCA1a-d) were cloned from human cells, expressed in COS or HEK cells and functionally characterised. Remarkably, in spite of its house-keeping nature, mutations in one of the two SPCA1-encoding alleles is only manifested as an autosomal dominantly inherited keratinocyte disorder known as Hailey-Hailey disease. Knock down of SPCA in zebrafish embryos leads to severe malformations (dorsalisation defects) and loss of pigmentation. With the apparent exception of fish, vertebrates express a related protein: SPCA2 (gene *ATP2C2*). SPCA2 expression is mainly confined to the GA from secretory cells in the gastrointestinal and respiratory tracts, prostrate, salivary and mammary gland. Both SPCA1 and SPCA2 can pump a single Ca^{2+} or Mn^{2+} per cycle and are nearly insensitive to thapsigargin. The Ca^{2+} affinity of the SPCAs is higher than that of the SERCAs but their catalytic turnover rate is lower.

Keywords: Golgi apparatus, secretory pathway, Mn^{2+}, Hailey-Hailey, SPCA

SERCA1 TRUNCATED ISOFORM (S1T) EXPRESSION MODIFIES ER/MITOCHONDRIA CALCIUM SIGNALING CROSS TALK AND IS INVOLVED IN ER STRESS-INDUCED APOPTOSIS

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By pumping calcium from the cytosol to the endoplasmic reticulum (ER), sarco/endoplasmic reticulum, calcium ATPase (SERCA) plays a major role in the control of calcium signaling. We previously described a SERCA1 variant (S1T) characterized by exon 11 splicing, encoding COOH terminally truncated protein (1). S1T protein is expressed in different human tissues and transformed cell lines and its overexpression in vitro has been demonstrated to: 1/ reduce ER calcium concentration by increasing ER calcium leakage; and 2/ induce apoptotic cell death. By using aequorin-based recombinant calcium probes specifically targeted to intracellular organelles, we show now that, as a consequence of ER calcium signaling alteration, S1T protein induces a reduction of agonist-evoked cytosolic calcium signals, without affecting capacitative calcium entry. In mitochondria, a drastic reduction of mitochondrial calcium uptake, a major alteration of mitochondrial structure network and an increase of organelle volume (swelling) were observed. Using subcellular fractions, western blot analyses and high resolution imaging, we found that S1T protein accumulates in the mitochondrial associated membranes (MAM) favouring contact sites between endoplasmic reticulum and mitochondria. Single cell calcium analyses, using ratiometric Pericam probe revealed an increase of mitochondrial calcium uptake in S1T cells that precedes mitochondrial swelling and calcium uptake reduction. We found that the mitochondrial structure alteration induced by S1T expression is calcium dependent since pre-treatment of S1T cells with BAPTA-AM (Ca^{2+} chelator) significantly reduced the extent of mitochondria alteration and corrected mitochondrial calcium concentration. We showed that endogenous S1T protein expression is induced both by ER stress drugs and by proteins known to induce ER stress in human pathological conditions (Hepatitis C virus core and cystic fibrosis mutated protein F508del). Finally, inhibition of endogenous S1T protein expression by transfection of specific RNAi allowed to prevent ER-stress-related deregulation of calcium signaling, alteration of mitochondrial structure and induction of apoptosis. This study shows for the first time that S1T protein plays a major role in the calcium signaling cross talk between ER and mitochondria and that, by this function, is involved in the control of ER stress-induced apoptosis.

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Keywords: Endoplasmic Reticulum (ER); mitochondria; calcium signaling; ER stress; mitochondrial swelling; ER-mitochondria contact sites

ANNEXIN A8 AFFECTS THE ORGANIZATION OF MULTIVESICULAR LATE ENDOSOMES

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Annexin A8 is a poorly characterized member of the annexin family of Ca^{2+}-regulated lipid binding proteins implicated in various aspects of membrane dynamics. Here, we used different approaches to analyze biochemical and functional properties of human annexin A8. We found that recombinant human annexin A8 can bind Ca^{2+}-dependently and with high specificity to phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P_2] and is capable of interacting with F-actin *in vitro*. In line with these characteristics annexin A8 is recruited to F-actin-associated PtdIns(4,5)P_2-rich membrane domains formed in HeLa cells upon infection with non-invading enteropathogenic *E. coli* (EPEC).
In non-infected HeLa cells, annexin A8-GFP is associated with the perimeter membrane of Lamp1/2-positive endosomes which contain lysobisphosphatidic acid (LBPA)-enriched internal vesicles, indicating that these are multivesicular late endosomes (MVEs). Overexpression of annexin A8-GFP leads to a more clustered perinuclear localization of the Lamp1-positive endosomes as well as an increase in the average MVE diameter and the appearance of individual "giant" endosomes. These observations were confirmed by cryo-immuno-EM. In contrast, RNAi-mediated depletion of endogenous annexin A8 causes a reduced average diameter of MVEs and a more dispersed distribution. Furthermore, the degradation of the activated EGF receptor is markedly impaired in annexin A8-depleted HeLa cells. Taken together, these data indicate that annexin A8 contributes to the correct morphological appearance, organization and function of the MVE compartment.

Keywords: Annexin A8, actin, late endosomes,LeLa cells, cryo-immuno-electron microscopy

SPECIFICITY, PROMISCUITY AND LOCALISATION OF ARF PROTEIN INTERACTIONS WITH NCS-1 AND PHOSPHATIDYLINOSITOL-4 KINASE-IIIβ

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ADP-ribosylation factor (ARF) proteins are small GTPases that act as key regulators of lipid modifying enzymes and transport vesicle coat assembly and thereby multiple intracellular vesicular transport pathways. Almost all studies to date have focused on the functions of either ARF1, a protein essential to intra-Golgi vesicular trafficking, Golgi to plasma-membrane transport and Golgi to endosome trafficking, or ARF6, a protein implicated in trafficking of cell surface receptors and phosphoinositide signaling at both the plasma-membrane and on endosomes. Little is known about the remaining ARF isoforms and although the mammalian ARF proteins share a high degree of sequence identity recent evidence has indicated that they may control distinct trafficking steps within cells (1). A major unanswered issue is the degree of specificity of ARF family members for different interacting proteins. To investigate potential functional differences between the human ARF proteins we have examined the interactions of all human ARF isoforms with two ARF1 binding proteins NCS-1 (2) and phosphatidylinositol-4 kinase-IIIβ (PI4Kβ). Use of a fluorescent protein fragment complementation method (3) showed direct interactions between ARFs 1, 3, 5 and 6 with NCS-1 but at different intracellular locations and a more specific interaction between ARFs 1 and 3 and PI4Kβ in live HeLa cells. These analyses suggest that in spite of their shared sequence homology, individual ARF proteins do exhibit selectivity for particular effectors.

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Keywords: Membrane, Traffic, Golgi, Specificity, Fluorescence, Complementation

EFFECT OF SAPONIN PERMEABILIZATION ON THE MORPHOLOGY OF THE ENDOPLASMIC RETICULUM AND THE GOLGI OF COS-1 CELLS.

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Selective permeabilization of the plasma membrane using saponin or digitonin is used to gain direct experimental access to intracellular compartments. The method is based on the fact that appropriately low concentrations of these substances do not act as detergents but mainly bind to cholesterol, thereby selectively permeabilizing the plasma membrane and leaving the permeability barrier of the cholesterol-poor intracellular membranes intact. This method has been proven very useful for the study of the properties of the Ca^{2+} pumps and Ca^{2+}-release channels present in the endoplasmic reticulum (ER), the Golgi and mitochondria. Because the preservation of functional pumps and channels does not imply the maintenance of higher-order structures, we now have studied the effect of saponin permeabilization on the morphology of the intracellular membranes.

The structure of the ER or Golgi was visualized in COS-1 cells transfected with either an ER-targeted or a Golgi-targeted cameleon (gift of Dr. R. Tsien). Permeabilization was carried out in a solution mimicking the cytosolic ionic composition. Treatment of COS-1 cells with a low concentration of saponin (10 μg/ml) resulted within a few minutes in a dramatic change of the structure of the ER. The delicate network seen in intact cells rapidly disappeared and was replaced by rounded vesicular structures that gradually increased in size, until after about 10 min little further change occurred. FRET measurements showed that these vesicles were capable of accumulating Ca^{2+} and releasing it in response to IP_3. The structural changes were partially prevented by the addition of liver cytosol supplemented with 100 μM GTP to the permeabilizing solution. Thus the maintenance of tubular ER structures in saponin-permeabilized cells requires cytosolic components and the presence of GTP, as has been observed for the *in vitro* establishment of a network-like structure from isolated ER vesicles[1] and for the integrity of the ER network in semi-intact Chinese hamster ovary cells[2]. Further work is needed to find out whether luminal continuity exists between the vesicular structures. The ER vesicles could correspond to the 'saposomes' released from saponin-treated hepatocytes in suspension[3]. The Golgi morphology as visualized with a Golgi-targeted cameleon was not appreciably affected by saponin treatment.

It is concluded that the interpretation of experiments on cells with disrupted plasma membrane, particularly if involving inter- or intra-organellar communication, should take into account the possibility of structural changes caused by loss of cellular components.

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Keywords: endoplasmic reticulum, Golgi, structure, permeabilized cells, saponin

THE ZEBRAFISH SPCA HOMOLOGUE IS A FUNCTIONAL CALCIUM/MANGANESE PUMP REQUIRED FOR EMBRYONIC DEVELOPMENT

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P-type Ca^{2+}-transport ATPases are key regulators of intracellular Ca^{2+} homeostasis. This study focuses on the SPCA (secretory pathway Ca^{2+} ATPase)-branch of the gene family. Its human homologues hSPCA1 and hSPCA2 are encoded by the *ATP2C1* and *ATP2C2* genes respectively. Both proteins are Ca^{2+}/Mn^{2+}-transport ATPases that localize to the Golgi apparatus of eukaryotic cells. While hSPCA1 is an ubiquitously expressed protein, hSPCA2 has a more restricted expression pattern limited to some secretory cell types. Mutations in the *ATP2C1* gene have been reported to cause Hailey-Hailey disease, a blistering skin disease.
We now have initiated the study of the role of SPCAs in Ca^{2+} homeostasis and embryonic development in the zebrafish *Danio rerio*. We report here the identification and functional characterization of the zebrafish SPCA orthologue. While in most teleost fish, many genes are represented by several paralogous copies, probably due to a genome duplication event, the zebrafish genome contains only one SPCA-isoform, termed zfSPCA.

The *zfSPCA* gene spans a genomic region of 60 kb and consists of 26 exons that after transcription, is translated into a 99 kDa protein. All intron/exon boundaries are conserved between *zfSPCA* and its human counterparts, illustrating their homology. We cloned the cDNA encoding zfSPCA and demonstrated that the corresponding protein overexpressed in COS-1 cells is a functional Ca^{2+}/Mn^{2+}-transporting enzyme. The protein forms a phosphorylated reaction intermediate that is dependent on Ca^{2+} or Mn^{2+}. Overexpressing COS-1 cells accumulated more Ca^{2+} and Mn^{2+} than control cells. This additional uptake was insensitive to the SERCA-specific inhibitor thapsigargin.
Morpholino-knockdown experiments demonstrate the essential nature of the *zfSPCA* gene in embryonic develepment. Morpholino-injected embryo's arrest in late embryonic development and present an obvious phenotype, characterized by severe defects in embryonic axis-formation (dorsalization) and lower jaw development. Moreover, these embryo's lack all pigmentation at 48 hours post fertilization.

We have demonstrated that the *zfSPCA* gene encodes a Golgi-localized functional Ca^{2+}/Mn^{2+}-transporting ATPase that is essential for embryonic development.

Keywords: Calcium, Manganese, ATPase, zebrafish, morpholino, SPCA

CHARACTERIZATION AND DETECTABILITY OF THE RAT HOMOLOGUE OF THE HUMAN NEUROENDOCRINE MARKER PROTEIN SECRETAGOGIN

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The recently cloned human EF-hand binding protein secretagogin has been established as a neuroendocrine cell marker. In this further work we describe the rat homologue, finding a considerable degree of sequence homology between human and rat genes. The anterior part of the rat protein (113aa) has 13 different residues when compared to its mouse counterpart. This peptide was produced recombinantly in *E.coli* and used for generation of a polyclonal rat secretagogin-specific mouse Antibody (Ab). Furthermore, mouse monoclonal Abs against the entire rat protein were also generated. Using these immunoreagents we performed immunohistochemistry in different rat tissues, finding out that this protein expression in rat is similar to that in humans and the highest levels are found in the pancreatic islets of Langerhans.

We also established a rat secretagogin-specific sandwich capture ELISA for detection of the protein in tissue culture fluid and rat serum. Stimulation of Rin-5F rat insulinoma cells with dexamethasone for 24 hours induced a 3-fold increase in secretagogin secretion, while reducing by two thirds insulin release. Other compounds known for influencing the beta cell secretory pathway (such as cAMP, PI3 K inhibitors, etc) were all found to regulate the secretion of secretagogin in an opposite manner to that of insulin.
In conclusion, we have characterized the rat homologue of secretagogin, a defined neuroendocrine marker, established a method for quantitatively measuring the protein in biological fluids and demonstrate an inverse correlation of insulin versus secretagogin secretion in rat insulinoma cells in culture.

Keywords: secretagogin, SCGN, calcium binding-protein

SECRETAGOGIN EXPRESSION IN TUMORS OF THE HUMAN BRAIN AND ITS COVERINGS

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Secretagogin is a recently described calcium-binding protein, which is expressed in a distinct subset of neurons of the human brain. In this study, we immunohistochemically analysed secretagogin expression in biopsies of 245 human intracranial neoplasms. We found focal or widespread secretagogin expression in tumour cells in 1/18 oligoastrocytomas, 2/20 anaplastic oligodendrogliomas, 1/4 clear cell and 1/5 classic ependymomas as well as 2/11 anaplastic ependymomas (1 of them clear celled), 2/10 glioblastomas, 3/11 gangliogliomas and 1/2 anaplastic gangliogliomas, 10/10 central neurocytomas, 5/10 classic medulloblastomas, 4/5 desmoplastic medulloblastomas, 3/5 large cell/anaplastic medulloblastomas, 3/5 neuroblastomas, 3/10 meningiomas, 2/10 haemangioblastomas, and 13/19 pituitary adenomas. In addition, we observed secretagogin expression in endothelial cells in 5/10 meningiomas, 2/5 haemangiopericytomas, and 2/10 haemangioblastomas. We detected no secretagogin expression in fibrillary astrocytoma, pilocytic astrocytoma, oligodendroglioma, dysembryoplastic neuroepithelial tumour (DNT), pineocytoma, pineoblastoma, subependymal giant cell astrocytoma (SEGA), atypical teratoid/rhabdoid tumour (AT/RT), or primary central nervous system lymphoma (PCNSL). We conclude that secretagogin is differentially expressed in human neuronal, glial, and embryonal brain tumours, meningial neoplasms and pituitary adenomas. Our findings suggest that secretagogin is involved in the calcium metabolism of a subset of neoplasms of the brain and its coverings.

Keywords: secretagogin; brain tumour; immunohistochemistry; neuropathology;

POTENTIAL ROLES OF CALPAIN7/PALBH IN ENDOSOMAL PATHWAY

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Calpain, a Ca^{2+}-dependent intracellular cysteine protease, is a biological modulator protease that catalyses the irreversible processing of substrate proteins. Extracellular calcium ions are concomitantly taken up by endocytosis and are released from endosomes during acidification caused by vacuolar ATPase. Most EF-hand proteins including calpains function as a Ca^{2+} sensing mediators by recognizing their target proteins. Calpain7/PalBH, one of the atypical calpains, was identified as an orthologue of *Aspergillus nidulans* PalB. Calpain7 contains two MIT (microtubule interacting and trafficking molecules) domains, a cysteine protease domain, a PBH (PalB homologous) domain and C2-like domain. PalB coordinates with Vps32 and regulates transcription as part of the organism's adaptive responses to alkaline environments, through the processing of the transcription factors. A human homologue of Vps32 (Vacuolar protein sorting32), CHMP4 (charged multivesicular body protein 4), is classified as a member of ESCRT (endosomal sorting complex required for transport), and we previously reported that CHMP4b and CHMP6 play roles in endosomal protein sorting pathway.

In this study, we demonstrate that calapain7 interacts with CHMP4c and CHMP1B and investigate the possibility that calpain7 plays roles in the endosomal pathway. Yeast two-hybrid assay demonstrated interactions of calpain7 with several ESCRT components involving CHMP4c and CHMP1B, which were mediated by MIT domains. Pulldown analysis revealed that StrEP-calpain7 interacts with FLAG-CHMP4c and FLAG-CHMP1B, respectively in HEK293T cells. By fluorescence microscopic analysis, overexpressed GFP-calpain7 in HeLa cells exhibited diffuse as well as a punctate distribution throughout the cytoplasm, and these fluorescent spots colocalized with FLAG-CHMP4c though they did not merge with signals of well-studied endosomal marker proteins such as EEA1 (early endosome antigen 1) and Lamp-1 (lysosomal membrane protein-1). GFP-calpain7 colocalized to aberrant endosomes caused by the overexpression of RFP-fused ATPase-defective dominant-negative form of SKD1/Vps4B (RFP–SKD1^{E235Q}). Furthermore GFP-calpain7 fluorescent spots partially colocalized with the endocytosed tetramethylrhodamine-EGF in HeLa cells at 10 min after EGF-uptaking.

Keywords: calpain, endosome, CHMP, ESCRT, MVB (multivesicular endosome)

Session 4 : Calcium channels

MODES OF ACTIVATION OF CRAC AND TRPC CHANNELS

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Activation of phospholipase C by G-protein-coupled receptors results in release of intracellular Ca^{2+} stores and activation of Ca^{2+} entry across the plasma membrane. Plasma membrane Ca^{2+} entry can result from any of a number of downstream consequences of phospholipase C activation, including changes in membrane phospholipid composition as well as the depletion of intracellular Ca^{2+} stores. In the latter case, the mechanism of entry has been referred to as *capacitative calcium entry* or *store-operated calcium (SOC) entry*. The best characterized electrophysiological current associated with capacitative calcium entry is the calcium-release-activated calcium current, or I_{crac}. A number of theories have been advanced for the mechanism of activation of SOC or CRAC channels, including conformational coupling with IP_3 receptors, a diffusible calcium influx factor (CIF) and more recently, an endoplasmic reticulum Ca^{2+} sensor, Stim1. The molecular nature of the SOC or CRAC channels is not known, but much attention has been focused on members of the TRP superfamily, particularly the sub-family known as TRPC. Ectopically expressed TRPC channels can clearly be activated by Ca^{2+} store depletion under certain experimental conditions. However, a major issue has been the failure of expressed TRPC channels to recapitulate the biophysical and pharmacological profile of I_{crac}. Recently, an integral plasma membrane protein, Orai1, has been described that is essential for I_{crac} in lymphocytes. We have found that in HEK293 cells, RNAi knockdown of either Stim1 or Orai1 significantly reduces SOC entry. Yet, overexpression of Stim1 was without effect, and overexpression of Orai1 inhibited entry. However, when we co-expressed these two proteins together, huge store-operated Ca^{2+} entry and store-operated Ca^{2+} currents were observed. These currents resembled I_{crac} in a number of ways, including a positive reversal potential, strong inward rectification, fast inactivation, and complete inhibition by 1 μM Gd^{3+} and 30 μM 2APB. Thus these two proteins appear to completely recapitulate both the activation mechanism and permeation mechanisms for I_{crac}. Stim1 appears to act by redistributing within a small component of the endoplasmic reticulum, approaching the plasma membrane, but does not appear to translocate into the plasma membrane.

Although TRPC channels do not recapitulate the expected biophysical properties of I_{crac}, they can show store-operated behavior under some experimental conditions. In a stable TRPC7-expressing HEK293 cell line exhibiting both store-operated and diacylglycerol-mediated TRPC7 activation, knockdown of Stim1 significantly inhibited store-operated but not diacylglycerol-mediated activity. Thus, TRPCs remain viable candidates for Stim1-regulated SOC channels, but likely play a role for channels that differ significantly in their properties from I_{crac}.

Keywords: CRAC, TRP channels, Ca-sensor, endoplasmic reticulum, Ca stores

STIM PROTEINS - MEDIATORS AND REGULATORS OF STORE-OPERATED CHANNELS

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The mechanism of coupling of store-operated channels (SOCs) has been a contentious issue for many years. The role of TRPC channels in this process has also been controversial. TRPC1 channels remain the most likely of the TRPC family to function as SOCs, yet their channel properties do not coincide with the known properties of SOCs. Recent RNAi screening approaches have revealed the single spanning membrane protein, STIM1, as being an essential component in the activation of SOCs. STIM1 is present in the ER and a luminal unpaired EF-hand functions as the sensor of Ca^{2+} to trigger SOC activation. STIM1 is also present in the PM and seems to play an important role in that membrane, perhaps by coupling to the store-operated channel itself. siRNA for STIM1 causes almost complete loss of SOC activity, but has no effect on TRPC3 or TRPC5 channel function. It has been proposed that store-depletion causes insertion of the STIM protein into the PM, however, we have not observed such translocation. Interestingly, the close structural STIM1 homologue, STIM2, has a profoundly different effect on SOC. Overexpression of STIM2 causes almost complete inhibition SOCs. In contrast, STIM2 has no effect on the function of TRPC3 channels. The STIM2 protein is expressed only in ER, and not in the PM. Store-depletion induces redistribution of ER STIM1 into distinct “puncta”, but does not alter distribution of STIM2 expressed alone. In contrast, when coexpressed with STIM1, STIM2 translocates into puncta upon store-depletion. Double-labeling shows exact coincidence of STIM1 and STIM2 within the puncta, and immunoprecipitation reveals direct interactions between STIM1 and STIM2. Independent of store-depletion, STIM2 is co-localized with and blocks the function of the D76AE87A- STIM1 EF-hand mutant that preexists in puncta and is constitutively coupled to activate SOCs. STIM2 appears to act within the puncta to interfere with the coupling between STIM1 and its downstream target in the activation of SOCs. The results support an “interactional” rather than “insertional” model for STIM1 in SOC activation. STIM1 appears to function as an essential “mediator” of SOC activation, while the role of STIM2 is likely as an important endogenous regulator of the function of STIM1. The functional and physical interactions between STIM proteins and TRPC channels is currently being investigated.

Keywords: STIM1, STIM2, TRPCs, EF-Hand

VOLTAGE-GATED CALCIUM CHANNELS IN GENETIC DISEASES

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Many properties of excitable cells rely on the activity of voltage-gated calcium channels (VGCCs). During the last decade, our understanding of how VGCCs gate and function has been illuminated by the identification and analysis of VGCC mutations responsible for a group of genetic diseases called “calcium channelopathies”. Calcium channelopathies include neuro-muscular, neurological, cardiac and vision syndromes. This great variety of disease traits further exemplifies the physiological importance of these VGCCs. The current challenge is to identify whether calcium channelopathies result not only from electrophysiological defects but also from defaults in the Ca_V/α_1 protein processing, including posttranslational modifications, quality control, trafficking and protein turn-over. Some recent data obtained in our laboratory on VGCC mutations involved in hypokalemic periodic paralysis (HypoPP1), episodic ataxia (EA2), childhood absence epilepsy (CAE) and Timothy syndrome (TS) will be presented. Patch-clamp studies, performed together with measurements of surface expression, trafficking and stability of the channel proteins have revealed the wide range of channel defects. Indeed, only a small fraction of VGCC mutations produce significant electrophysiological changes. Some VGCC mutations impair protein synthesis and membrane targeting (i.e. $Ca_V2.1$ in EA2). We further describe that truncated forms of $Ca_V2.1$ proteins associated with EA2 exert a dominant negative, providing a likely explanation for the dominant negative transmission of this disease. Conversely, we also report examples of VGCC mutations that enhance expression at the plasma membrane (i.e. $Ca_V3.2$ in CAE). Overall, functional analyses of VGCC mutations offer important new insights into VGCC function and provide a more comprehensive view of the pathogenesis of the corresponding human disorders. Ultimately, understanding of these channel mutations should lead to a better diagnosis and possibly treatment of hereditary diseases in humans.

Keywords: VGCCs, disease, channelopathies, ataxia

REGULATION OF NADPH OXIDASE BY EXTRACELLULAR Ca^{2+} IN MYELOID CELLS

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In myeloid cells, Ca^{2+} influx across the plasma membrane is largely mediated by Ca^{2+}-store depletion through the store-operated Ca^{2+} entry (SOCE) [1]. This particular mechanism seems to be a crucial event for the NADPH oxidase activation in response to N-formyl-L-Methionyl-L-Leucyl-L-Phenylalanine (fMLF) [2,3]. By a double labelling fluorescent approach [4-5], we have investigated the Ca^{2+} conditions underlying the oxidative response in neutrophil-like dimethylsulfoxide-differentiated HL-60 cells (≠HL-60 cells). ≠HL-60 cells responded to a fMLF stimulation by a free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) elevation (363 ± 30 nM) followed by an early H_2O_2 production (31.1 ± 4.0 A.U.) reflecting the NADPH oxidase activation. Both responses were strongly reduced either when reported inhibitors of SOCE, SK&F 96365 [6] and 2-aminoethoxy diphenyl borate [7] were applied in the medium, or in the absence or in chelation of extracellular Ca^{2+}. In this last case, early Ca^{2+} readdition after fMLF stimulation permitted to restore almost full $[Ca^{2+}]_c$ elevation and H_2O_2 production (respectively 86 ± 6 % and 67 ± 11 % of restoration at 5 s) showing that extracellular Ca^{2+} has to be present at the initiation of the response to authorize a full NADPH oxidase activation.

TRPC channels have been suggested to support SOCE in a variety of cell types. By real-time PCR, we have screened the TRPC gene products in order to establish their expression pattern in purified human neutrophils and to validate ≠HL-60 cells and PLB-985 cells as neutrophil models for the study of TRPC functions in relation to NADPH oxidase activation. TRPC1, TRPC3, TRPC4 and TRPC6 mRNAs were detected in all tested cell types. TRPC3 and TRPC4 were found to be the foremost expressed TRP channels in neutrophils and TRPC3 was dominant in HL-60 and PLB-985 cells.

These results suggest that TRPC channels may be largely responsible for the fMLP-induced calcium-dependent NADPH oxidase activity in myeloid cells.

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Keywords: NADPH oxidase, extracellular Ca^{2+}, neutrophils, myeloid cells.

DIRECT EVIDENCE FOR A ROLE OF TRPC6 IN HUMAN HEPATOMA CELL PROLIFERATION

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External calcium is essential for cell proliferation but the nature of the calcium channels involved in the process is probably tissue-dependent. We have previously shown [1] using carboxyamidotriazole and 2-APB, that Hep G2 and Huh-7 human hepatoma cell proliferation is dependent on capacitative calcium entry (CCE). Human hepatoma cell lines only express TRPC1 and TRPC6 and, in this work, we have investigated whether TRPC6 plays a role in cell proliferation. First, TRPC6 expression was increased or decreased by transient transfection of Huh-7 cells with plasmid encoding for the protein or ShRNA respectively. Specific antibody against TRCP6 was used in western blots to monitor expression in both conditions. CCE amplitude was directly related to the level of TRPC6 expression. CCE increased by 125 % in over-expressing cells and decreased by 85 % in ShRNA-transfected cells. In order to measure both CCE and cell proliferation, we then developped two stable clones of Huh-7 cells over or down expressing TRPC6. In over-expressing Huh-7 stable clones, CCE amplitude and rate of cell proliferation were increased by 160% and 50% respectively. We observed the opposite results in Huh-7 stable clones transfected with a plasmid encoding for the ShRNA against TRPC6. Namely, CCE amplitude was reduced by 40 % and proliferation rate decreased by 65 % in ShRNA stably transfected clones as compared to control cells. It is not clear yet whether the difference in CCE decreased amplitude between transiently and stably transfection results from a lower inhibition of TRPC6 expression in stable clones or the presence of TRPC1 in these cells. However, we can conclude that TRPC6 plays an important role in the control of CCE amplitude and rate of cell proliferation in human hepatoma cells.

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Keywords: Liver, calcium channels, cancer, TRPC

T-TYPE $Ca_v3.3$ CALCIUM CHANNELS PRODUCE SPONTANEOUS LOW THRESHOLD ACTION POTENTIALS AND INTRACELLULAR CALCIUM OSCILLATIONS

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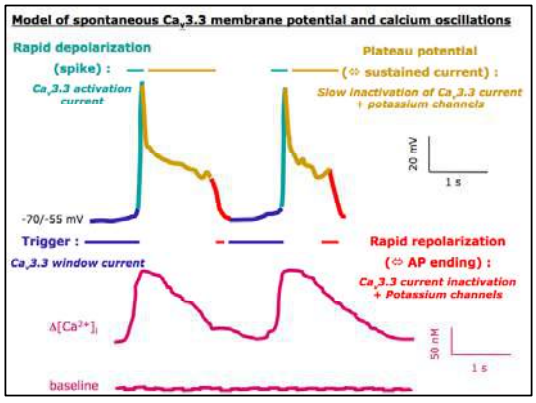
Many physiological processes involve the ubiquitous second messenger calcium. Above all the kind of signals described for calcium signaling, intracellular calcium concentration ($[Ca^{2+}]_i$) oscillations constitute a specific signal. Although the major description of this phenomenon concerns oscillations linked to internal sources of calcium (after the activation of endoplasmic reticulum), we describe (Chevalier et al., 2006) spontaneous oscillations of intracellular calcium from external sources. These oscillations are the consequence of the spontaneous opening of a membrane T-type $Ca_v3.3$ voltage-gated calcium channels isotype in a range of potential corresponding to the window current properties of these channels. They are associated with membrane potential oscillations and the calcium signals intrinsic to the oscillations have variable properties (in frequency, amplitude and duration) closely linked to the variable shape of action potential. We describe here the specific functional properties of $Ca_v3.3$ channels in generating plateau potential consecutive to a complex inactivating/deactivating current, associated to a sustained $Ca_v3.3$ current after the activation current. The duration of the plateau potential, and consequently of the action potential, is dependent of the threshold potential from which they are generated. Overall, the data demonstrate that the $Ca_v3.3$ window current is critical in triggering intrinsic electrical and $[Ca^{2+}]_i$ oscillations. The functional expression of $Ca_v3.3$ channels can generate spontaneous low threshold calcium action potentials through its window current, attesting that $Ca_v3.3$ channels can play a primary role in pacemaker activity.



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Keywords: $[Ca^{2+}]_i$ oscillations, $Ca_v3.3$ T-type calcium channel, window current, pacemaker.

EFFECTS OF *TRANS*- AND *CIS*-RESVERATROL ON AGONIST AND K^+-INDUCED INCREASES IN Ca^{2+} IN VASCULAR SMOOTH MUSCLE CELLS

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We have investigated the effects of the *trans*- and *cis*- isomers of the natural polyphenol resveratrol (*t*-RESV and *c*-RESV) on agonist- and K^+-induced increases of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) using fura-2 AM and patch-clamp techniques in isolated vascular smooth muscle cells (A7r5). Arginine vasopressin (AVP, 0.5 μM) caused a transient elevation in $[Ca^{2+}]_i$ (increase in $[Ca^{2+}]_i$ = 552.8 ± 16.6 nM) followed by a sustained rise (103.5 ± 9.2 nM) that was partially inhibited by nifedipine (~30%) and completely abolished by Ni^{2+}. According to our previous reports[1], pre-incubation with *t*-RESV or *c*-RESV caused a sustained elevation in basal $[Ca^{2+}]_i$. In these conditions, both the transient and the sustained rise elicited by the subsequent application of AVP (0.5 μM) were significantly reduced (AVP-induced peak in the presence of 30 μM *t*-RESV or *c*-RESV: 262.7 ± 38.2 nM and 297.8 ± 33.2 nM, respectively; AVP-induced plateau in the presence of 30 μM *t*-RESV or *c*-RESV: 60.9 ± 5.1 nM and 88.6 ± 7.9 nM, respectively; $P < 0.05$ with respect to control values). When the isomers were applied on the plateau phase of the AVP-induced response, a biphasic change in $[Ca^{2+}]_i$ was observed: first, a reduction of the plateau during the first 5 min (32% and 21% of reduction for 30 μM *t*-RESV and *c*-RESV, respectively), followed by an increase (starting after ~10 min) until it reaches similar values to those of the agonist-induced plateau. Similar results were obtained for both isomers on angiotensin II-induced increases in $[Ca^{2+}]_i$. In Ca^{2+}-free high K^+ (60 mM) depolarizing medium, reintroduction of $CaCl_2$ (1.5 mM) caused a sustained rise in $[Ca^{2+}]_i$ (maximal value: 120.4 ± 14.2 nM). Pre-incubation of the cells with *t*-RESV or *c*-RESV (10, 30 μM) significantly reduced this high K^+-induced $[Ca^{2+}]_i$ response (59.0 ± 4.6 nM and 63.2 ± 7.2 nM for 30 μM *t*-RESV and *c*-RESV, respectively; $P < 0.05$ with respect to control). When the isomers were administrated during the high-K^+-induced sustained increase in $[Ca^{2+}]_i$, they caused a biphasic response: first, a reduction of $[Ca^{2+}]_i$ (40% and 28% for 30 μM *t*-RESV and *c*-RESV, respectively), followed by an increase (15% and 19% for 30 μM *t*-RESV and *c*-RESV, respectively). In whole-cell clamped cells, *t*-RESV and *c*-RESV inhibited a voltage-dependent sustained L-type Ca^{2+} current. The % of inhibition reached with 30 μM *t*-RESV or *c*-RESV were 66.2 ± 7.2 and 42.1 ± 4.4, respectively. Our results suggest that both *t*-RESV and *c*-RESV exert a biphasic effect on agonist- and K^+-induced changes in $[Ca^{2+}]_i$ in rat aortic myocytes: 1) an inhibitory effect (probably due to a blockade of L-type Ca^{2+} channels) which may explain, at least in part, the endothelium-independent vasorelaxant effects exhibited by both resveratrol isomers in isolated rat aortic rings[2] and 2) a potentiation which is probably due to a previously described release of intracellular Ca^{2+} and activation of extracellular Ca^{2+} entry[1].

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Keywords: resveratrol, vascular smooth muscle cells, calcium, arginine-vasopressine, Ca-transport.

INDUCTION OF ER STRESS BY BLOCKAGE OF T-TYPE CALCIUM CHANNELS IN SYMPATHETIC NEURONS

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The endoplasmatic reticulum (ER) plays a major role in Ca^{2+} signaling and storage in the cells. Alterations in ER Ca^{2+} levels may result in the accumulation of misfolded proteins, triggering ER stress and a series of adaptative responses designed to restore functionality, including the unfolded protein response (UPR). UPR generally involves a shutdown of protein synthesis, an enhancement of protein degradation and the concerted upregulation of multiple ER chaperones. If the stress stimuli is severe and/or sustained, the cells' fate is put on the balance and specific death programs can be activated (Kim et al., 2006 for review).
Voltage-gated Ca^{2+} channels (VGCCs) mediate Ca^{2+} entry in response to membrane depolarization which, in turn, controls membrane excitability, synaptic transmission and gene expression leading to the activation of survival programs, among other neuronal processes (Catteral, 2000 for review). In the present work, we have investigated whether particular types of VGCCs may also contribute towards neuronal ER Ca^{2+} homeostasis.
We first demonstrate that application of micromolar concentrations of T-type channel blockers triggers the death of dissociated rat superior cervical ganglion neurons (SCGs). However, at sub-lethal concentrations of the blockers the cells undergo a slow neurodegenerative process, which may last several days and is reversible upon wash-out of the drugs. Second, we have checked by RT-PCR that the SCGs express the Cav3.2 and Cav3.3 isoforms of T-type channels, previously unreported in these neurons. Third, we have found by RT-PCR and immunoblotting that aplication of T-type channel blockers induces UPR as seen by the up-regulation of ER chaperones. Finally, we show by immunofluorescence and electron microscopy that the UPR which is unleashed by the blockage of T-type channels, is linked to the development of a macroautophagic process.
In conclusion, our work shows that the blockage of T-type channels induces ER stress in unstimulated sympathetic neurons, thus revealing a vital contribution of these low-threshold activated Ca^{2+} channels to ER Ca^{2+} homeostasis at the resting membrane potential.

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Keywords: T-type channel, Ca^{2+}homeostasis, ER stress, Unfolded Protein Response, Autophagy

EFFECTS OF STIM1 INVALIDATION ON ER CALCIUM REFILLING EFFICIENCY

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Changes in the intracellular free Ca^{2+} concentration regulate many cellular functions such as cardiac contraction, hormone secretion, gene transcription, and cell death. In non-excitable cells, Ca^{2+} signals are generated by the controlled release of Ca^{2+} from the endoplasmic reticulum (ER), and the depletion of the ER, in turn, activates store-operated Ca^{2+} channels (SOC) at the plasma membrane. The mechanism that activates SOC channels in response to ER depletion has long been elusive. Recently, STIM1 (STromal Interaction Molecule 1), a single transmembrane protein (Williams, Manji et al. 2001) localize either at the ER or plasma membrane (PM) has been proposed to function as an ER Ca^{2+} sensor that activates store-operated calcium channels (Liou, Kim et al. 2005; Roos, DiGregorio et al. 2005). The supposed mode of action of STIM1 is either a translocation from the ER to the plasma membrane or a direct interaction between STIM1 located at ER with STIM1 located at the PM (Zhang, Yu et al. 2005; Spassova, Soboloff et al. 2006). To assess the Ca^{2+} signaling role of STIM1, we used genetically encoded yellow cameleon Ca^{2+} indicators (YC) to measure Ca^{2+} changes in the cytosol, the endoplasmic reticulum and in the mitochondrial matrix in HeLa cells. In this study, we focused on the effect of STIM1 invalidation on the efficiency of ER Ca^{2+} refilling.

Cells were co-transfected with YC together with dsRNAs and Ca^{2+} responses measured at the single-cell level by Ca^{2+} imaging. The transfection of HeLa cells with dsRNA targeting STIM1 mRNA resulted in 75% of STIM1 mRNA invalidation. In STIM1 silenced cells, the thapsigargin induced Ca^{2+} influx was inhibited by about 73%, while the amplitude of the Ca^{2+} signal measured in Ca^{2+} free medium was not altered, indicating that Ca^{2+} release from the ER was not affected by STIM1 invalidation. The ER Ca^{2+} refilling mechanism were investigate with a protocol where Ca^{2+} stores were completely depleted by three consequent histamine stimulations in Ca^{2+} free medium, then agonist was washed prior the re-addition of 2 mM Ca^{2+}. In order to estimate the level of Ca^{2+} stores refilling, cells were re-stimulate with histamine in Ca^{2+} free medium. In STIM1 invalidated cells, the Ca^{2+} influx recorded in the cytosol during Ca^{2+} re-addition period was completely abolished but surprisingly the last histamine stimulation elicited a large cytosolic Ca^{2+} augmentation. Moreover, on the contrary to control cells, in STIM1 silenced cells, increasing Ca^{2+} concentration up to 50 mM during the Ca^{2+} re-addition period did not enhance the cytosolic influx. Direct ER Ca^{2+} measurements confirmed that STIM1 silencing did not prevent the ER Ca^{2+} refilling. Namely, in STIM1 invalidated cells despite no visible Ca^{2+} influx in the cytosol, ER Ca^{2+} stores were able to refill efficiently. Preliminary observations suggest that the ER Ca^{2+} refilling is not achieved through mitochondria, as no Ca^{2+} uptake is measured in mitochondria following Ca^{2+} re-addition in STIM1 silenced cells.

Thus, our data show that even under condition of reduced Ca^{2+} entry the ER refilling is largely preserved. We propose that the “silent Ca^{2+} influx” occur through Ca^{2+} micro-domains that result from close interactions between the plasma membrane and the ER.

Keywords: STIM1, Ca2+ influx, ER Ca2+ refilling, micro-domains, yellow cameleon

TWO TYPES OF CALCIUM ENTRY PATHWAYS COEXIST IN A HUMAN UMBILICAL VEIN ENDOTHELIAL CELL LINE

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Although the Ca^{2+} influx in non-excitable cells, such as endothelial cells, is of great importance for their physiology, neither the molecular identity of the channel supporting this influx, nor the mode of activation are well understood. Among the diversity of mechanisms proposed that leads to Ca^{2+} entry, two major pathways have emerged. One pathway requires the presence of an agonist that, upon receptor activation, produces second messenger(s) that subsequently opens a Ca^{2+} permeable ion channel. Based on its proposed mechanism of activation, this Ca^{2+} entry is referred to as receptor-activated Ca^{2+} entry (RACE). The other pathway is directly linked to the Ca^{2+} filling state of the endoplasmic reticulum (ER), and is called capacitative Ca^{2+} entry or store-operated Ca^{2+} entry (SOCE). The non-requirement of an agonist for SOCE activation is in a sense artificial, as physiologically, store depletion is the result of agonist-induced Ca^{2+} release. Nevertheless, activation of SOCE can be achieved in the absence of any agonist, and thus profoundly differs from the RACE. The aim of this study is to differentiate the type of Ca^{2+} entry pathways in endothelial cells and to characterize them. We measured Ca^{2+} changes both in the cytosol (Fura-2) and in the ER ($D1_{ER}$) in order to monitor Ca^{2+} influx and to evaluate the importance of store depletion, respectively. In addition, patch clamp experiments were performed on endothelial cells derived from human umbilical vein (Ea.hy926), to obtain an electrophysiological signature of the currents supporting the different influxes.
By stimulating the cell either with an agonist (histamine) or by passively depleting the ER by SERCA inhibitor (thapsigargin, TG), together with the use of several known Ca^{2+} entry blockers, we were evaluating the appearance of different pathways leading to Ca^{2+} entry. We found that La^{3+} and SK&F96365 have opposite effects depending on the protocol used to stimulate Ca^{2+} entry. La^{3+} inhibits to a great extent TG-induced calcium entry whereas SK&F96365 preferentially blocks the histamine-stimulated calcium entry. Moreover, after inhibiting SOCE by La^{3+}, histamine was still able to stimulate Ca^{2+} entry, thus pointing to the existence of two distinct Ca^{2+} influx pathways in endothelial cells. Interestingly, the level of store deletion during histamine stimulation was about 20% of what is achieved following passive store depletion, and thus is in agreement with the minor involvement of SOCE during physiological cell stimulation. The whole-cell currents activated by histamine or TG presented the same sensitivity to inhibitors as found with Ca^{2+} imaging experiments. To better characterize the channels involved in Ca^{2+} entry, we also performed single channel recording. In cell-attached configuration, histamine stimulated a non-selective cationic channel of 20-25 pS. This channel was not activated by store depletion, which is in favour of the existence of different type of channels supporting various influxes in endothelial cells.

Keywords: calcium influx, endothelial cells, endoplasmic reticulum, electrophysiology.

GATING OF KCNQ1 BY Ca^{2+} AND VOLTAGE

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The slow I_{ks} K^+ channel plays a major role in repolarizing the cardiac action potential. The I_{ks} channel is composed of KCNQ1 and KCNE1 subunits. KCNQ1 subunits form the essential domains of the I_{ks} K^+ channel, including the pore, the selectivity filter and the voltage sensor. KCNE1 subunits are ancillary, modulating I_{ks} K^+ channel properties. The KCNQ1 C-terminus contains two binding sites for calmodulin (CaM). CaM binding is essential for I_{ks} channel assembly and gating[1]. Here, we used the *Xenopus* oocytes expression system to record KCNQ1-mediated current from inside-out macropatches at different intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$). The results showed that an increase in $[Ca^{2+}]_i$ from 10^{-8} to 2×10^{-7}M produced a negative shift in the half-maximal voltage of KCNQ1 current activation by 22mV. KCNQ1/KCNE1 channel activation appeared even more Ca^{2+}-sensitive, and respective increase of $[Ca^{2+}]_i$ shifted the half-maximal voltage of current activation by 50mV. Application of the CaM antagonist W-7 to KCNQ1 or KCNQ1/KCNE1 channels strongly inhibited the current amplitude. The W-7 inhibitory effects were rapid and reversible. These data indicate that CaM confers Ca^{2+} sensitivity to the KCNQ1 channel. When $[Ca^{2+}]_i$ was rapidly increased or decreased at depolarized membrane potentials, I_{ks} channel amplitudes responded accordingly by a rapid increase or decrease, respectively. The time courses of the Ca^{2+}-responses were within the time frame of a single action potential. These results show that the exquisite Ca^{2+} sensitivity of the I_{ks} channel was fully reversible and independent of ATP and protein kinase activity. Our findings provide a basis for understanding the Ca^{2+} sensitivity reported for cardiac I_{ks} currents at high stimulation rates. In addition, we identified mutations in the KCNQ1 pore region which perturbed or completely attenuated the Ca^{2+} sensitivity of the KCNQ1 channel. This suggests that the KCNQ1 gating is controlled by both voltage and Ca^{2+}, reminiscent to the gating of BK channels.

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Keywords: Calmodulin, Ca^{2+}, KCNQ1, potassium channel, KCNE1

Ca^{2+} HOMEOSTASIS DYSREGULATION IS ASSOCIATED WITH TRIMETHYLTIN-INDUCED DEGENERATION IN CULTURED RAT HIPPOCAMPAL NEURONS

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We have previously shown that hippocampal neurons expressing the calcium-binding protein calretinin are selectively spared by the neurotoxicant trimethyltin (TMT), possibly owing to calcium-buffering action against calcium overload which may accompany cell death(1,2). The present study aimed to determine whether TMT-induced degeneration is correlated with dysregulation of intracellular Ca^{2+} homeostasis. In cultured rat hippocampal neurons loaded with Fluo 4-AM changes in cytosolic Ca^{2+} concentrations were studied by confocal Ca^{2+} imaging and measured as $\Delta F/F$ ratio. Administration of TMT induced a time-dependent and concentration-dependent $[Ca^{2+}]_i$ increase in most cells studied. Cell pre-treatment with the selective SERCA pump blocker, thapsigargin, significantly reduced the TMT-induced $[Ca^{2+}]_i$ increase, thus indicating that Ca^{2+} release from intracellular stores significantly contributes to the effects studied. When cells were perfused with Ca^{2+}-free solutions, TMT produced $[Ca^{2+}]_i$ increases that, during the first 25 min, were significantly smaller than those found in the presence of extracellular Ca^{2+}. However, after 40-min drug exposure, $\Delta F/F$ values observed with or without extracellular Ca^{2+} were not significantly different. In the presence of nifedipine, a selective blocker of L-type Ca^{2+} channels, the effects of TMT were similar to those obtained in Ca^{2+}-free solution, suggesting that Ca^{2+} entry through L-type Ca^{2+} channels contributes to the TMT-induced $[Ca^{2+}]_i$ increase, at least in the early phase of the drug's action. Cell pre-treatment with the intracellular Ca^{2+} chelator BAPTA-AM, counteracting calcium overload, significantly reduced the neurotoxic effects of exposure to TMT. These data indicate that the neurotoxic action of TMT is likely due to Ca^{2+} homeostasis dysregulation produced via changes in both Ca^{2+} release from intracellular stores and Ca^{2+} influx through the plasma membrane.

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Keywords: TMT, calcium, neurodegeneration,hippocampus

A NEW INHIBITOR OF STORE OPERATED CALCIUM INFLUX, THE BTP2, REDUCES CALCIUM SIGNALING AND CALCIUM DEPENDENT FUNCTIONS OF NEUTROPHILS AND HL60 CELLS.

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The store operated calcium entry (SOCE) constitutes a key factor in signaling pathways which control human neutrophil functions. SOCE is mediated by plasma membrane ion channels which could be potential therapeutic targets. To better understand SOCE function, we used a new SOCE inhibitor which has been recently developed in T cells: the 3,5-bistrifluomethyl pyrazole derivative (BTP2) [1,2,3]. We investigated how this compound could act on differentiated HL60 cells and on human neutrophils. Firstly, our results showed that 10 µM of BTP2 reduced significantly SOCE in both cell types by at least 80% when calcium influx was induced by Thapsigargine (TG) or by fMLF, a chemotactic peptide. Secondly, our results showed that BTP2 acted also when its concentration was reduced to 0,2 µM with a incubation time increased from 5 min to 24h. Interestingly, this action was irreversible when BTP2 was washed out 3h before the experiment. Neutrophils are cells of the innate immune system. We evaluated the impact of BTP2 on their calcium dependent functions, like β-glucosaminidase secretion and superoxide anion production which were reduced by 30% and 82% respectively. However BTP2 did not significantly inhibit the bactericidal function of phagocytosis and *P. aeruginosa* killing. The molecular targets of BTP2 are not yet well known, but some studies suggest that BTP2 acts directly on some SOC channels, in particular the TRPC channels [4]. We studied TRPC expression in granulocytes, but their composition differs during the differentiation. Indeed, undifferentiated cells express mRNA of only TRPC7, when differentiated HL60 cells expressed in addition TRPC1 and neutrophils TRPC1 and 3. We also investigated whether these changes of gene expression are correlated with a change of BTP2 impact on store operated calcium influx. Our preliminary results suggest that after activation of cell with TG, 0,2 µM of BTP2 reduced SOCE by 30% in differentiated HL60 cells and neutrophils, whereas in undifferentiated HL60 cells 1 µM BTP2 was needed to affect calcium influx. Furthermore, we investigated the effect of BTP2 on the proliferation of leukemia HL60 cells. Our results indicate that this proliferation was reduced significantly by 15 µM BTP2. We currently investigate how BTP2 acts on HL60 cells, and in particular if it induces their entry into apoptosis. In conclusion, BTP2 appears also like a new tool to investigate the role of calcium signaling in granulocytes. And in the perspective to develop a new therapeutic tool, the properties of BTP2 are encouraging to find compounds that inhibit either the harmful neutrophil functions or the leukaemia development without damaging antibacterial host defence.

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Keywords: Neutrophils, Calcium signaling, SOCE, TRPC channels, Cell activation, Proliferation.

Session 5 :
Calcium binding proteins and pathophysiology

CALMODULIN-DEPENDENT KINASES AS THERAPEUTIC TARGETS

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A primary goal of biomedical research is to improve health by preventing and treating disease. This requires the development of new and effective therapies, the vast majority of which are bioavailable, low molecular weight chemicals. Despite a large increase in funding for biomedical research, there has been a sustained downward trend for bringing new therapies to the bedside over the past few decades. The theoretical goal of single target-based discovery that currently dominates drug discovery and development is to develop drugs affecting a single gene product in order to selectively treat a disease and minimize unwanted side effects. It is ironic, then, that major reasons for the recent high failure rate in drug development are problems associated with safety or lack of efficacy. Among the contributors to the failure rate are problems associated with molecular properties, or those attributes that make a chemical drug-like, and the dissociation of *in vitro* ligand development from *in vivo* explorations of pathophysiology. Recently, we have explored three alternative but complementary approaches to drug discovery. Two of these approaches are being explored by focusing on a set of closely related protein kinases regulated by calcium and calmodulin: myosin light chain kinase (MLCK) and death associated protein kinase (DAPK). Common to both approaches is the use of integrative chemical biology, or the development and use of small molecule compounds *in vivo*, to explore the potential for modulating the progression of disease-relevant pathophysiology. This presentation will be a research-in-progress discussion on the attenuation of tissue injury by bioavailable inhibitors of these two calcium/calmodulin-regulated protein kinases.

Keywords: disease, MLCK, calmodulin, drug discovery.

INFLAMMATORY ACTIVITIES OF MYELOID RELATED PROTEIN 8 (MRP8, S100A8) AND MRP14 (S100A9).

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Myeloid related protein 8 (MRP8, S100A8) and MRP14 (S100A9) are two abundant cytoplasmic proteins expressed in phagocytes. Both proteins associate to complexes which are released during activation of phagocytes by a so far unknown pathway of secretion. To identify novel functions of these S100-proteins we established a MRP14 -/- mouse. Interestingly these mice lack also MRP8 at protein level probably due to instability of this protein in the absence of its binding partner. We demonstrate that mice lacking MRP8/MRP14 complexes are protected from endotoxin-induced lethal shock. MRP8/MRP14 promote endotoxin-triggered stress responses of phagocytes. MRP8 is the active component of the complex. MRP8 activates a classical signaling cascade involving intracellular translocation of MyD88, activation of IRAK-1 and DNA-binding activity of NF-kB. Activation of this signaling pathway results in elevated expression of inflammatory molecules, e. g. tumor necrosis factor. Analysing phagocytes obtained from Toll-like-receptor 4 (TLR4) -/- mice we demonstrate that MRP8 interacts with this receptor complex thus representing a novel endogenous activator of TLR4-signaling. MRP8/MRP14 also exhibit pro-inflammatory effects on endothelial cells inducing expression of adhesion molecules, cytokines, pro-thrombotic molecules, and increasing endothelial permeability via break up of cell-cell junctions. These mechanisms are known to play a role in the pathogenesis of sepsis and lethal endotoxin-induced shock. Thus, MRP8/MRP14 complexes are novel inflammatory molecules which play an important role in the pathogenesis of inflammatory reactions.

Keywords: S100A8, S100A9, Calgranulin, Calprotectin, Sepsis

MOLECULAR BASIS OF CALCINEURIN FUNCTION IN DROSOPHILA MUSCLE DEVELOPMENT

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Calcineurin is a calcium-activated protein phosphatase composed of a catalytic subunit (calcineurin A), a regulatory subunit (calcineurin B), and the calcium-binding protein calmodulin. The serine/threonine-specific phosphatase functions within a signaling pathway that regulates biological responses in a variety of cell types, including cardiac and skeletal muscle cells. Genes encoding the calcineurin subunit proteins are highly conserved across animal species as the Drosophila genome contains three canA and two canB genes that are 75% and 88% homologous to their vertebrate relatives, respectively (1). Our recent studies have demonstrated a critical requirement for calcineurin in Drosophila indirect flight muscle (IFM) formation (2, 3). The IFM serves as a valuable system to delineate gene functions in muscle growth and myofiber-type utilization. Results thus far have demonstrated regulatory and functional interactions among genes encoding canB2 and the myosin heavy chain (mhc) protein: canB2 mutants present with an IFM hypercontractile phenotype, canB2 alleles show genetic interactions with mhc alleles, and mhc expression is greatly diminished in canB2 mutants. A calcineurin-responsive transcriptional enhancer for the mhc gene is being characterized to identify the calcineurin-regulated transcription factor(s) controlling mhc expression in the IFM. These combined approaches should generate novel information on the genetic and molecular control of calcineurin signal transduction in Drosophila muscle.

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Keywords: Calcineurin, Drosophila genetics, Indirect flight muscle, Myosin heavy chain, Transcriptional control

VISININ-LIKE- PROTEIN-1 (VILIP-1) IS EXPRESSED IN PANCREATIC β-CELLS AND REGULATES INSULIN SECRETION AND INSULIN GENE TRANSCRIPTION

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Visinin-like protein-1 (VILIP-1) is a member of the neuronal Ca2+-sensor (NCS) family that modulates Ca^{2+}-dependent cell signaling events. VILIP-1, which is primarily expressed in the brain, increases cAMP formation in neurons by modulating adenylyl cyclase (AC), but its functional role in other tissues remains largely unknown. In the present study, we demonstrated that VILIP-1 was expressed in murine pancreatic islets and β-cells. To gain insight into the functions of VILIP-1 in β-cells, we used both overexpression and siRNA knockdown strategies. VILIP-1 was shown by immunocytochemistry to be expressed in β-cells and colocalized with insulin. Glucose-stimulated insulin secretion (GSIS) was significantly enhanced in MIN6 cells and isolated mouse islets overexpressing VILIP-1 by 49±6% or 35±8.5% ($p<0.05$, $n=10$) respectively. However, VILIP-1 overexpression had no effect on basal insulin secretion, insulin content or preproinsulin mRNA levels. Interestingly, cAMP accumulation in VILIP-1-overexpressing MIN6 cells was increased by 43±3.8% and 41.3±6.7% ($p<0.05$, $n=9$) at basal and stimulatory glucose, while the PKA inhibitor H89 attenuated the increase in GSIS, suggesting that VILIP-1 mediated enhancement in GSIS is cAMP-PKA associated. Since cAMP potentiates insulin secretion by various means including through direct action on exocytotic machinery, cell capacitance was measured in dispersed mouse islets. These measurements demonstrated that VILIP-1 overexpression led to 120±30% increase in cell capacitance indicating an enlargement in the readily releasable pool (RRP) and therefore the stimulation of exocytosis. Conversely, reduction of VILIP-1 expression (85±2%) by siRNA duplex targeting this protein, with a decrease in the accumulation of cAMP (26.3±4.1% at basal glucose and 27±6.5%, at stimulated glucose, $p<0.05$) produced an increase in basal insulin secretion (214±9.6%) and GSIS (49±4.5%). These changes were accompanied by significant increases in total insulin content (66±0.05%) and preproinsulin mRNA (54±14.7%). A rat insulin I promoter driving luciferase assay showed the expression of luciferase was elevated in VILIP-1 knockdown cells, suggesting the activity of insulin promoter was enhanced hence insulin gene transcription was upregulated in these cells. And this is possibly related to the upregulated expression of the insulin transactivator, Pdx-1 (by 46±14.7%) revealed by real-time PCR. And H89 treatment can not block these effects, indicating a cAMP-PKA independent pathway. Conclusively, we have demonstrated for the first time that VILIP-1 is expressed in pancreatic β-cells and modulates GSIS as well as insulin gene transcription. At physiological level, VILIP-1 has inhibitory effect on insulin gene transcription in pancreatic beta cells via regulation of Pdx-1 in a cAMP-PKA independent pathway. In contrast, when overexpressed, VILIP-1 enhanced glucose induced insulin secretion by acting on exocytosis in a cAMP-associated manner.

Keywords: Visinin-like protein 1, insulin secretion, insulin gene transcription, cyclic AMP

S100B/RAGE-DEPENDENT ACTIVATION OF MICROGLIA : STIMULATION OF COX-2 EXPRESSION AND TNF-α RELEASE

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The Ca^{2+}-modulated protein, S100B, is secreted into extracellular fluids and found in serum thereby affecting cellular activities in a paracrine, autocrine and endocrine manner (1,2). Astrocytes release S100B constitutively, and increases in S100B release occur upon astrocyte stimulation with a number of agents (1,2). At the concentration found in the brain extracellular space under normal conditions S100B mostly acts as neurotrophic factor promoting neuronal survival and neurite outgrowth (1,2). Thus, S100B might be important during development and the initial phases of brain insults as a protective agent towards neurons. Trophic effects of S100B on neurons depend on binding to the receptor for advanced glycation end products (RAGE) (1,2). However, on accumulation in the brain extracellular space S100B may cause astrocyte and neuronal apoptosis (1,2). Neuronal apoptosis caused by high doses of S100B results from chronic stimulation of RAGE activity (3) and upregulation of RAGE expression in neurons (4), and it might be consequent to S100B-dependent stimulation of iNOS expression and activity in astrocytes and microglia (1,2). S100B was shown to bind to and activate RAGE in microglia (5), but S100B's ability to stimulate iNOS in microglia proved independent of RAGE transducing activity, though dependent on the density of RAGE extracellular domain on the microglial surface (6). We show here that binding of S100B to RAGE in BV-2 microglia results in the upregulation of expression of the pro-inflammatory cyclo-oxigenase 2 (COX-2) via Ras-Cdc42-Rac1-dependent activation of c-Jun NH_2 terminal protein kinase (JNK) and Ras-Rac1-dependent stimulation of NF-κB transcriptional activity. The S100B/RAGE pair also stimulates NF-κB transcriptional activity via a Ras-MEK-ERK1/2 and a Cdc42-Rac1-p38 MAPK pathway with resulting upregulation of IL-1β expression and stimulation of TNF-α secretion. Also, inhibition of JNK results in negation of S100B's ability to stimulate TNF-α secretion, and neutralization of culture medium TNF-α with a specific antibody results in reduction of S100B-induced upregulation of COX-2 expression. Thus, the S100B/RAGE pair might independently upregulate COX-2 expression via activation of the JNK and NF-κB pathways and via Ras-MEK-ERK1/2- and Cdc42-Rac1-p38 MAPK-dependent stimulation of TNF-α secretion with autocrine TNF-α-dependent upregulation of COX-2 expression. Given the crucial roles of COX-2 and TNF-α in the inflammatory response, we propose that, by engaging RAGE, extracellular S100B might play a role in microglia activation in the course of brain insults.

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Keywords: S100B, microglia, inflammation, brain, COX-2, TNF-α

NUCLEAR Ca^{2+}/CALMODULIN-DEPENDENT PROTEIN KINASE II IN THE MURINE HEART

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Ca^{2+} signaling through CaMKII is critical in regulating myocyte function with regard to excitation-contraction-relaxation cycles and excitation-transcription coupling. To investigate the role of nuclear CaMKII in cardiac function, transgenic mice were designed and generated to target the expression of a CaMKII inhibitory peptide, AIP (KKALRRQEAVDAL), to the nucleus. The transgenic construct consists of the murine α-myosin heavy chain promoter followed by the expression unit containing nucleotides encoding a four repeat concatemer of AIP, a nuclear localization signal (NLS), and a FLAG epitope. The CaMKII inhibitor domain has a sequence encoding a tetramer of the CaMKII autocamitide inhibitory peptide (AIP_4). The 13-amino acid sequence (AIP) is known to be a highly specific and potent inhibitor of CaMKII (1 and 2). Western blot and immunohistochemical analyses demonstrate that AIP_4 is expressed only in the nucleus of cardiac myocytes of the transgenic mice (NLS-AIP_4). The function of cytoplasmic CaMKII is not affected by the expression of AIP_4 in the nucleus. Inhibition of nuclear CaMKII activity is assayed by analyzing the translocation of histone deacetylase 5 (HDAC5) from nucleus to cytoplasm. It has been demonstrated that this translocation occurs following phosphorylation of HDAC5 by activated CaMKII (3). We observed reduced translocation of HDAC5 from nucleus to cytoplasm in perfused NLS-AIP_4 mouse hearts compared to wild type mouse hearts stimulated with ET-1, indicating that the nuclear CaMKII activity is inhibited in NLS-AIP_4 mice. Inhibition of nuclear CaMKII activity causes NLS-AIP_4 mice to have smaller hearts than their nontransgenic littermates. With physiological stresses such as pregnancy or aging (8 months), NLS-AIP_4 mice develop enlarged atria, systemic edema, sedentariness, and morbidity. Our results suggest that absence of adequate calcium signaling through nuclear CaMKII regulated pathways leads to development of cardiac disease.

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Keywords: CaMKII, heart, nucleus.

BIOCHEMICAL FEATURES OF PRESENILINS AND THEIR INVOLVEMENT IN Ca^{2+} SIGNALING OF LYMPHOBLASTS FROM ALZHEIMER'S DISEASE PATIENTS

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Key pathogenic factors in Alzheimer's disease (AD) include two presenilin proteins (PS1 and PS2), membrane components of γ-secretase complexes responsible for generation of toxic β-amyloid peptides. Pathogenesis of AD is also associated with deregulation of Ca^{2+}-signalosome and we analyzed points of cross-talk between presenilins and Ca^{2+} signaling. In particular, we studied the interaction of PS2 with Calmyrin1 (CaMy1), an EF-hand Ca^{2+} binding protein from calmyrin family similar to Neuronal Calcium Sensors. Immunocytochemical stainings of rat brain with antiPS2 and antiCaMy1 antibodies and immunoblotting of subcellular fractions demonstrated some overlap of CaMy1 and PS2 in neurons. We also found high amounts of both endogenous CaMy1 and PS2 in human immortalized lymphocytes which are easily accessible and we investigated interaction of both proteins in lymphocyte membranes. Affinity chromatography on recombinant CaMy1 cloned from lymphocyte cDNA, pull-down and mass spectroscopy methods demonstrated that CaMy1 binds specifically to the C-terminal fragment of PS2 and not to PS1 in Ca^{2+}-independent manner. Ca^{2+}-independence of this interaction suggests that CaMy1 might transduce Ca^{2+} signal to PS2 already in preformed CaMy1/PS2 complexes, similarly as shown for troponin complexes. In addition, demonstrated separation of PS2 from PS1 by CaMy1-affinity sustains the proof of principle for purification of endogenous PS1 and PS2. Moreover, we also shown that these two highly homologous presenilins can be efficiently separated from each other by ion-exchange chromatography. These two independent method isolating PS2 from PS1 indicate that despite their homology PS1 and PS2 may exist in different membrane pools and in variegated γ-secretase complexes.

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Keywords: presenilins, Alzheimer's disease, calmyrin, EF-hand proteins, Ca2+-signaling

DIFFERENT Ca^{2+} SIGNALING PATHWAYS MEDIATED BY CELLULAR PRION PROTEIN INTERACTION TO LAMININ γ-1 PEPTIDE OR STRESS-INDUCIBLE PROTEIN 1

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The cellular prion protein (PrP^c) is a highly conserved cell surface glycoprotein expressed in the Central Nervous System. Its conformationally modified isoform, PrP^{sc}, is responsible for the Transmissible Spongiform Encephalopathies or prion diseases. In the last years, multiple and diverse functions of PrP^c have been reported. In order to elucidate the physiological functions of PrPc, our group identified and characterized some partners for this protein. PrP^c binds to the carboxi-terminal of the Laminin γ-1 chain (residues 1575-RNIAEIIKDI-1584) and to the Stress Inducible Protein 1 (STI 1). PrP^c-STI1 binding induces neuronal differentiation and survival in wild-type ($Prnp^{+/+}$) hippocampal neurons while no effect was observed in cells from PrP^c-gene ablated ($Prnp^{0/0}$) mice. Herein, we demonstrated that the γ-1 peptide was able to induce an increase in the percentage of cells extending neurites and in the number of neurites/cell in wild-type ($Prnp^{+/+}$) but not in ($Prnp^{0/0}$) neurons. Using an intracellular Ca^{2+} probe, Fluo- 3 AM, we verified that γ-1 peptide, in particular the KDI domain, increased intracellular Ca^{2+} both by endoplasmic reticulum mobilization and extracellular medium influx. Conversely, γ-1 chain peptide presented no effect in Ca^{+2} signaling in $Prnp^{0/0}$ neurons. The role of Ca^{2+} signaling in the γ-1 peptide-mediated neuritogenesis was evaluated in hippocampal neurons after treatment with PLC or PKC inhibitors. These drugs were able to abrogate neurite extension and the PLC inhibitor blocked the Ca^{2+} signaling in neurons treatet with γ-1 chain peptide. We also evaluated the role of PrP^c-STI1 interaction on Ca^{+2} signaling and our data demonstrate that STI1 induced an intracellular Ca^{2+} increase only by extracellular medium influx. Thus, our data indicate that neuritogenesis induced by PrP^c engagement to the KDI domain at the γ-1 chain peptide, is dependent on intracellular Ca^{2+} signaling by mobilization intracellular stores and by extracellular medium influx. On the other hand, STI1-induced Ca^{2+} signaling is dependent on extracellular medium influx. Therefore, through its interaction with two distinct partners, laminin and STI1, PrP^c can trigger different calcium signaling pathways which may lead to diverse biological functions.

Keywords: Cellular Prion Protein, Calcium, Laminin, STI 1

ZINC INDUCES TAU HYPERPHOSPHORYLATION BY THE MEANS OF GLYCOGENE SYNTHASE KINASE

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Alzheimer disease (AD) is neuropathologically characterised both by extracellular amyloid plaques and by neuronal-paired helical filament constituted of aggregates of hyperphosphorylated tau proteins. We have previously reported in AD an over-expression of S100A6, a protein that binds zinc with a particular high affinity [1]. This protein was pathologically expressed in astrocytes in the vicinity of the amyloid plaques, where zinc is abnormally concentrated (1mM). The high zinc concentration in the amyloid plaques and the affected permeability of the neuronal membranes at this location should allow a non-specific transfer of zinc from the extracellular space to the neurons. We hypothesized that the abnormal zinc concentration find in the amyloid plaques could then interfere with transductions pathways leading to tau phosphorylation. The effect of zinc on tau phosphorylation was studied on the clonal CHO line expressing the 1N4R human tau isoform. The addition of 100 to 250µM zinc sulphate on CHO cells showed by immunoblotting a clear increase of tau phosphorylation. The hyperphosphorylation of tau could then be partly explained by GSK-3 beta phosphorylation of its active tyrosine 216. By immunohistochemistry we could observe an increase in tau hyperphosphorylation especially when using PHF1 antibody, specifically recognizing abnormal phosphorylated tau epitopes. These results suggest that the abnormal extracellular zinc concentration present in the neocortex in AD could play a role in neuronal tau hyperphosphorylation.

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Keywords: Alzheimer, Amyloid, Tau protein, GSK-3-b, Zinc

FACTOR Xa AND THROMBIN EVOKE ADDITIVE CALCIUM AND PROINFLAMMATORY RESPONSES IN ENDOTHELIAL CELLS SUBJECTED TO COAGULATION

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Endothelial cells react to factor Xa and thrombin by proinflammatory responses. However, it is unclear how these cells respond under physiological conditions, where the serine proteases factor VIIa, factor Xa and thrombin are all simultaneously generated, as in tissue factor-driven blood coagulation. We studied the Ca^{2+} signaling and downstream release of interleukins (ILs), induced by these proteases in monolayers of human umbilical vein endothelial cells. In single cells, factor Xa, but not factor VIIa, complexed with tissue factor, evoked a greatly delayed, oscillatory Ca^{2+} response, which relied on its catalytic activity and resembled that of SLIGRL, a peptide specifically activating the protease-activated receptor 2 (PAR2). Thrombin even at low concentrations evoked a rapid, mostly non-oscillating Ca^{2+} response through activation of PAR1, which reinforced the factor Xa response. The additive Ca^{2+} signals persisted, when factor X and prothrombin were activated in situ, or in the presence of plasma that was triggered to coagulate with tissue factor. Further, thrombin reinforced the factor Xa-induced production of IL-8, but not of IL-6. Both interleukins were produced in the presence of coagulating plasma. In conclusion, under coagulant conditions, factor Xa and thrombin appear to contribute in different and additive ways to the Ca^{2+}-mobilizing and proinflammatory reactions of endothelial cells. These data provide first evidence that these serine proteases trigger distinct signaling modules in endothelium that is activated by plasma coagulation.

Keywords: calcium signaling, coagulation, endothelial cells, factor Xa, thrombin

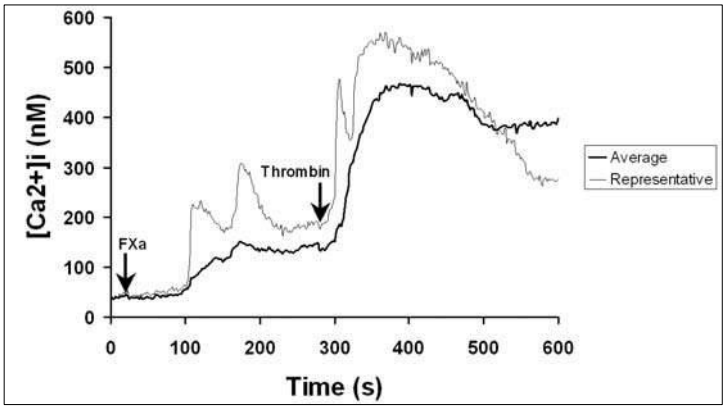

Fig.1. Additive calcium signals in HUVEC stimulated with factor Xa (40nM), followed with a stimulation with thrombin (10nM)

INHIBITION OF S100B EXPRESSION IN ASTROCYTES RESULTS IN REDUCED PROLIFERATION AND IN STELLATION: IMPLICATIONS FOR ASTROCYTE ACTIVATION

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The Ca^{2+}-modulated protein of the EF-hand type, S100B, has been proposed to have a role in neuroprotection and neurodegeneration, protecting neurons against stress- or β-amyloid-induced apoptosis, stimulating neurite outgrowth and inhibiting neurotoxin-induced microglia activation, at relatively low doses (1-5) and, at high doses, causing neuronal apoptosis via engagement of the receptor for advanced glycation end products (5) and stimulation of NO production and release of pro-inflammatory cytokines in astrocytes and microglia (6-10). Thus, participation of S100B in brain cell trophism, degeneration and inflammatory processes seems to be a matter of extracellular effects of the protein. By contrast, little is known about the functional role(s) of S100B within astrocytes in relation to their proliferation and participation in inflammation. In the normal brain tissue astrocytes show a stellate shape, and upon brain injury they become polygonal, proliferate and migrate thereby participating in reactive gliosis. To start addressing the role of S100B in astrocytes, we analyzed GL15 astroglioma cells for effects of silencing of S100B. Astrocytes treated with S100B siRNA proliferated and migrated less than mock-silenced astrocytes and tended to acquire a stellate shape with concomitant stress fiber collapse and filopodia formation. It is known that astrocyte stellation is dependent on inactivation of the RhoA-ROCK axis (11). Treatment of astrocytes with a sub-maximal (1 μM) dose of the ROCK inhibitor, Y27632, caused a fraction of astrocytes to undergo changes reminiscent of stellation although not frank stellation, while the majority of astrocytes treated with S100B siRNA plus Y27632 showed stellation. The shape changes observed in S100B siRNA-treated astrocytes were associated with decreased activation of the mitogenic kinase, Akt, an activator of the RhoA-ROCK axis (12). Thus, silencing of S100B might result in ROCK inactivation and, hence, in stellation and reduced proliferation and migration, suggesting that S100B might normally facilitate the RhoA/ROCK activity via stimulation of Akt thereby favoring proliferation and migration. As alterations of the blood-brain barrier results in retraction of astrocytic processes and in astrocyte proliferation and migration, S100B might contribute to these events in case of brain insults. Also, S100B might contribute to astrocyte proliferation and migration during brain development, while its accumulation in astrocytes might contribute to their neoplastic transformation via activation of the tumorigenic Akt.

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Keywords: S100B, astrocytes, stellation, RhoA/ROCK, inflammation, proliferation.

ZINC- AND HEPARIN-BINDING PROPERTIES OF S100A12

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The inflammatory-associated proteins S100A8, -A9 and -A12 form a subgroup of S100s known as calgranulins. Though there are amino acid sequence similarities between them, S100A12 has distinct roles. In addition to Ca^{2+} many S100 proteins also bind Zn^{2+}, which plays a functional role in the apoptotic and antimicrobial activities of the S100A8/A9 heterodimer (1). However the antifilarial activity of S100A12 is not affected by Zn^{2+} (2) and the functional significance of S100A12 Zn^{2+}-binding is unclear. S100A8 and -A9 interact with heparan sulphate moieties on endothelial cells (3), but potential glycosaminoglycan (GAG) binding properties of S100A12 have not been described. In solution, S100 proteins exist as homodimers, or heterodimers with other S100 proteins whilst S100A8 and -A9 can also form oligomeric complexes in the presence of Ca^{2+}.

The present study was conducted to analyze S100A12 heparin binding properties and metal ion- induced complex formation. Chemical cross-linking and SDS-PAGE revealed S100A12 is dimeric in the presence of physiological Ca^{2+} concentrations, however a hexamer was formed in the presence of Zn^{2+} with or without Ca^{2+}, but was not induced by Cu^{2+}. Compared with apo-S100A12, Zn^{2+} and Ca^{2+} enhanced 1-anilino-8-naphthalene sulfonic acid fluorescence to a similar extent but were not additive when combined, suggesting both ions induce a similar change in tertiary structure. S100A12 bound heparin Sepharose and eluted with ~500 mM NaCl indicating a relatively high affinity binding. S100A12 activated THP-1 macrophages to express interleukin 8. The role of Zn^{2+}-binding in this activity was tested by both addition of free Zn^{2+} and depletion by a specific chelator. Responses were diminished by chelation, but were not altered by addition of free Zn^{2+}. Treatment of THP-1 cells with heparanase, or activation in the presence of heparin did not alter IL-8 induction. Like other chemokines, localisation of S100A12 on the extracellular matrix by GAG binding may enhance its chemotactic gradient for monocytes. The functional relevance of the Zn^{2+}-induced S100A12 complex is under investigation.

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Keywords: S100, S100A12, zinc, heparin, hexamer

cGMP-DEPENDENT KINASE (cGK) CONTROLS CALCIUM SIGNALING IN T HELPER 2 TYPE LYMPHOCYTES

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T helper (Th) lymphocytes include Th2 and Th1 cells that produce interleukin (IL)-4 and interferon (IFN)γ, respectively. Th2 cells are implicated in allergic diseases such as asthma. T cell receptor (TCR)-associated signaling pathways differ between Th2 and Th1 cells. We previously showed that Th2 but not Th1 cells selectively expressed dihydropyridine (DHP) sensitive Ca^{2+} influx at play in the Ca^{2+} response elicited by TCR stimulation (1). DHP-sensitive Ca^{2+} channels have been described in B lymphocytes, and shown to be regulated by cGK (2).

Therefore we explored a role for this kinase in Ca^{2+} signaling and Th2 cytokine production. Ovalbumin (OVA)-specific transgenic T lymphocytes were differentiated along the Th2 pathway by repeated stimulations with OVA, antigen presenting cells, IL-4 and anti-IFNγ antibodies (abs). We showed that TCR stimulation induced a 2-fold-increase in the cGMP level. NO donors, that activate guanylate cyclase (GC) and generate cGMP, or cell permeant-cGMP induced an increase in intracellular Ca^{2+}, nuclear translocation of the Ca^{2+}-regulated transcription factor NFAT and *Il4* gene transcription (3). All these effects were abolished by the DHP antagonist nicardipine, showing that cGMP regulates, directly or not, DHP-dependent Ca^{2+} influx. cGMP may activate cGK or interfere with cAMP-dependent signaling through activation or inhibition of phosphodiesterases (PDE), depending upon the type of PDE. Pharmacological inhibitors of GC and of cGK suppressed Ca^{2+} signaling, and IL-4 synthesis by Th2 cells, whereas inhibitors of PDE or of cAMP-dependent kinase had no effect.

We then analyzed the capacity of T lymphocytes from cGK1-/- mice to differentiate into Th2 cells and to produce IL-4. Th cells from cGK1-/- or control littermates were polyclonally stimulated with anti-TCR and anti-CD28 abs once a week for 3 weeks. The addition of IL-4 plus anti-IFNγ abs or IL-12 plus anti-IL-4 abs skewed towards Th2 and Th1 cell development respectively. Ca^{2+} signaling and the synthesis of IL-4 by Th2 cells from cGK1-/- mice were impaired, while IFNγ synthesis by Th1 cells was spared.

These results indicate that cGK regulates Ca^{2+} signaling in Th2 cells, which can be a therapeutic target in the management of allergic diseases, the frequency of which is increasing in Western Countries.

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Keywords: T lymphocytes; allergy; IL-4; cGMP-dependent kinase; dihydropyridine-sensitive Ca^{2+} influx

BLOCK OF GLUTAMATE-, NICOTINE- AND ELECTRICALLY STIMULATED CALCIUM RESPONSES BY PARKINSON AND ALZHEIMER DRUGS.

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The antimuscarinic drugs procyclidine and biperiden have been useful in the symptomatic treatment of Parkinson's disease. The mechanism of action is not known. The drugs also inhibit NMDA receptors (NMDAr) and the antiglutamatergic properties, together with the antimuscarinic effects, were found useful in restricting brain seizures resulting from nerve agent poisoning. The block of NMDAr, however, has not been characterized.
We characterized the antiglutamatergic and antinicotinic properties of procyclidine and biperiden as well as memantine (an Alzheimer drug) and gacyclidine (GK11, a novel NMDAr antagonist) with calcium imaging techniques in primary cultures of cerebellar granule neurons (CGN) from rat and in cocultures of CGN with astrocytes. Procyclidine and biperiden have similar in vivo potencies for neuroprotection but, surprisingly, the in vitro IC50 for block of CGN NMDAr by biperiden (about 15 μM) is much larger than that of procyclidine (1μM). This discrepancy is explained by the significant difference in pharmacokinetics of the drugs.
Additional possible drug targets of calcium regulation were investigated. The drugs all blocked calcium responses mediated by nAChr channels (α7, α4β2, α2β3) in the human neuronal cell line SH-SY5Y. The IC50s were in the low μM range, i.e. with potencies as low as block of NMDAr. In CGN we also found that procyclidine blocks voltage activated calcium channels (P/Q or L-type), albeit with lower potency (IC50 35 μM).
The effects of electrical stimulation, NMDA or glutamate on CGN viability were studied. Pulsed electrical stimulation gave rise to a calcium influx that was blocked by all drugs. Electrically stimulated calcium responses, however, desensitized over minutes and did not give rise to excitoxicity, whereas NMDA and glutamate did. Surprisingly, although all drugs gave neuroprotection against NMDA only gacyclidine was effective against glutamate.

Keywords: NMDA, PCP, Parkinson, nAChr, organophosphate, epilepsia

EFFECT OF S100A9 EXPRESSED AT THE CELL SURFACE ON THE DIFFERENTIATION OF GLANDULAR CELL CARCINOMAS

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[Purpose] Lung cancer is a leading cause of death in men and women worldwide. Lung cancers are devided by histopathology, and adenocarcinomas constitute almost half of lung cancer cases. Calcium-binding protein S100A9 is expressed in lung adenocarcinoma and closely associated with tumor differentiation, showing a higher correlation with poor differentiation[1]. S100A9 expression is also associated with the poor differentiation of breast, liver and thyroid carcinomas[2-4]. We investigate S100A9 in cultured human lung and breast carcinoma cell lines to know (1) intracellular localization of the protein, (2) any association with other S100 family members and (3) the role of S100A9 on tumor invasion and metastasis.
[Methods] Human breast carcinoma MCF-7 and lung adenocarcinoma PC-14 and LC-2/ad, poorly and moderately differentiated, respectively, cells are cultivated as described[2]. Localization of S100A9 is determined by Western blotting and immunocytochemistry. Cells are treated with antisense oligonucleotide[5] to suppress the gene expression of S100A9.
[Results] S100A9 was expressed in the membrane fraction of breast and lung carcinoma cells and immunostained without Nonidet P-40 permeablization. S100A8, which forms heterodimer with S100A9 in neutrophils and monocytes, was not expressed in the lung cells. A condition to fully suppress S100A9 gene expression by antisense oligonucleotide is under investigation.
[Discussion] Of interest is that S100A9 knocked-out cells would have an ability to invade and metastasize.

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Keywords: S100A9, breast carcinoma, lung carcinoma, antisense ologinucleotide

NOX5 : A Ca^{2+}-REGULATED NADPH OXIDASE

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NADPH oxidase 5 (NOX5), is a homodimeric protein expressed in lymphoid, spleen and testis. As the other NOX isoforms, NOX5 conserves the NADPH, FAD and heme-binding sites required for the electron transport; but it differs from the others for its unique N-terminus which contains three canonical EF-hands motifs. NOX5 was shown to generate superoxides in response to intracellular Ca^{2+} elevations (Banfi *et al,* 2001).
To study the Ca^{2+}-dependent mechanism activation of NOX5 by its N-terminus we produced the recombinant regulatory domain (residues 1-169), called NOX5-EF. Flow dialysis experiments show that NOX5-EF contains four specific Ca^{2+} -binding sites. Point mutagenesis enable us to identify the fourth non-canonical EF-hand. Ca^{2+} binding to NOX5-EF causes marked conformational changes, with the exposure of a hydrophobic patch, that allows NOX5-EF to bind a model peptide for calmodulin targets. We also demonstrate with pull down experiments that after Ca^{2+} binding the regulatory N-terminus domain directly interacts with the catalytic C-terminus domain, leading to the activation of the enzyme (Banfi *et al,* 2004). So our study allows us to formulate a new activation mechanism in the NADPH oxidases and Ca^{2+}-activated enzymes: Ca^{2+}-induced conformational changes of NOX5 N-terminus lead to the activation of the enzyme through an intramolecular interaction between the regulatory and the catalytic domain of the enzyme. We are in the process of identifying the binding site in the catalytic domain.

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Keywords: NADPH oxidase, superoxide, Ca^{2+}-binding proteins, Ca^{2+}-regulated protein interaction, Ca^{2+}-signaling, EF-hand.

BIOPHYSICAL CHARACTERIZATION OF S100A8 AND S100A9 IN THE ABSENCE AND PRESENCE OF BIVALENT CATIONS

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S100A8 (myeloid related protein 8, MRP8) and S100A9 (MRP14) belong to the S100 family of EF-hand calcium-binding proteins and are expressed in a tissue-and cell-specific manner. Both proteins are released during activation of phagocytes by a so far unknown mechanism suggesting both intra- as well as extra-cellular functions. Common to all S100 proteins is their tendency to dimerize. In addition, an increasing focus of research is placed on the importance of higher oligomeric structures of S100 proteins induced by bivalent cations due to the hypothesis of assembling receptors (1). Recently we have shown that a tetramer composed of two molecules S100A8 and S100A9 is formed in the presence of calcium and that tetramer formation is at least dependent on a functional C-terminal EF-hand (2, 3). Furthermore we could show that the tetramer is a prerequisite for physiological function such as bundling of tubulin filaments (3, 4).
In addition to Ca^{2+} S100A8 and S100A9 are able to bind Zn^{2+} with high affinity. It could be shown already that extracellular S100A8/S100A9 exhibits antimicrobial activity just by chelation of Zn^{2+}, which is necessary for growth of bacteria (5). However, little is known about oligomeric structures of S100A8/S100A9 induced by Zn^{2+}. We here demonstrate by biophysical methods (MALDI-MS, ESI-MS and fluorescence spectroscopy) that Zn^{2+} is also able to induce S100A8/S100A9 tetramers. Using mass spectrometric investigations we demonstrate that Zn^{2+} triggers the formation of $(S100A8/S100A9)_2$-tetramers by Zn^{2+}-specific binding sites rather than by interactions with Ca^{2+}-specific EF-hands. The Zn^{2+}-induced tetramer is structurally very similar to the Ca^{2+}-induced tetramer. Thus we propose that beside Ca^{2+}, Zn^{2+} acts as a regulatory factor in S100A8/S100A9-dependent signaling pathways.

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Keywords: S100A8, S100A9, Calprotectin, Zinc, Tetramer

BINDING OF THE VIRAL IMMUNOGENIC OCTAPEPTIDE VSV8 TO NATIVE GLUCOSE- REGULATED PROTEIN GRP94 (GP96) AND ITS INHIBITION BY ATP AND Ca^{2+}

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The molecular chaperone Grp94 (gp96) of the endoplasmic reticulum lumen plays an essential role in the structural maturation and/or secretion of proteins destined for transport to the cell surface. Its proposed role in binding and transferring peptides for immune recognition is, however, a controversial issue. We have here studied by surface plasmon resonance (SPR) spectroscopy the interaction of native glycosylated Grp94 at neutral pH and 25^{o}C with the viral immunogenic octapeptide RGYVYQGL (VSV8), derived from vesicular stomatitis virus nucleoprotein (52-59). The peptide binds reversibly with low affinity ($[A]_{0.5}$ ~640 μM) and a hyperbolic binding isotherm, and the binding is partially inhibited by ATP and Ca^{2+} at concentrations that are present in the ER lumen, and the effects are explained by conformational changes in the native chaperone induced by these ligands. Our data present experimental support for the recent proposal that VSV8 under native conditions binds to Grp94 by an adsorptive, rather than a bioselective, mechanism, and thus further challenge the proposed *in vivo* peptide acceptor-donor function of the chaperone in the context of antigen-presenting cell activation.

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Keywords: ATP; Cations; Grp94; SPR; VSV8

REDOX MODIFICATIONS OF INTRACELLULAR PROTEINS INDUCED BY STIMULATING THE CELLS WITH EXTRACELLULAR S100B

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S100B protein belongs to a multigenic family of Ca2+-modulated proteins of the EF hand type expressed in high abundance in the nervous systems. It also plays a role in other tissues like adipocytes, melanocytes, chondrocytes, skin Langerhans cells, lymphocyte subpopulations and skeletal muscles cells [1,2]. A number of intracellular targets of S100B have been identified [3]. In addition to its intracellular functions, S100B is transported outside the cell and possesses extracellular activities [1,2]. At relatively low (nM) levels extracellular S100B exerts trophic effects i. e. protects neurons against neurotoxic agents and stimulates neurite outgrowth. On the other hand, high (uM) concentration of extracellular S100B is toxic to the cells. The switch between trophic and toxic effects of extracellular S100B is not fully understood. Among the different results of stimulating the cells with S100B there is enhanced activity of nitric oxide synthase (production of reactive nitrogen species, RNS) and/or production of reactive oxygen species, ROS [4,5]. A strict balance of intracellular production of RNS and ROS has been shown to be important for physiological function, while disturbing this equilibrium leads to cell toxicity. At the same time there is accumulating evidence that the most important consequences of RNS and ROS production are covalent modifications of intracellular proteins which play a role in signal transduction (S-nitrosylation) or lead to toxic effects (i.e. tyrosine nitration, formation of cysteine sulphonic acid) [6].

The aim of this work is to study the changes of redox modifications of intracellular proteins induced upon stimulating cells with extracellular S100B. Such work should test our hypothesis that, in such biological system, protein S-nitrosylation is accompanying the well studied phosphorylation in signal transduction. Of special interest for us are the changes in cellular “redox proteome” correlated with S100B interaction with its extracellular receptor RAGE.
Differences in the cellular “S-nitrosome’, as detected using mass spectrometry based proteomic analysis will be presented for cell lines both expressing and not expressing receptor RAGE, which were stimulated with either trophic or toxic concentration of extracellular S100B.

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Keywords: S-nitrosylation, S100B

Session 6 : Systems biology

FROM CALCIUM CALMODULIN TO GENE EXPRESSION

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The protein phosphatase, calcineurin, was the first protein phosphatase whose activity was shown to be dependent on calcium and calmodulin. It was therefore ideally suited to play an important role in triggering cellular responses to the second messenger, calcium. More than ten years ago the identification of calcineurin as the target of the immunosuppressive drugs, cyclosporin A (CsA) and tacrolimus (FK506) revealed its key role in the transduction pathway from the plasma membrane to the nucleus leading to T cell activation**.** Since then, this pathway has been shown to play a critical role in cellular processes as diverse as the development and function of the nervous system, muscle differentiation, embryogenesis, angiogenesis, and ion homeostasis. Because of its multiple sites of action, alteration of calcineurin activity has been implicated in the pathogenesis of cardiac hypertrophy, congenital heart diseases, and immunological and neurological disorders.

Keywords: calcineurin, immunosuppressive drugs, cell differentiation

CALCIUM RELEASE THROUGH INOSITOL 1,4,5-TRISPHOSPHATE RECEPTORS IS REQUIRED FOR THE INDUCTION OF CARDIAC HYPERTROPHY

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Heart disease is a major cause of mortality in the developed world. In 2004 it was responsible for 137,700 deaths in the UK (24% of all deaths). A major predictor of mortality from heart failure is cardiac hypertrophy. Cardiac hypertrophy is characterised by an increase in the muscle mass of the heart due to cellular enlargement without any proliferation. Under physiological conditions, hypertrophy is an adaptive response to an increased haemodynamic load. Under pathological conditions, such as hypertension, viral infection or due to mutations in genes encoding sarcomeric proteins, hypertrophy progresses to dilated cardiomyopathy, arrhythmias, and death.

Multiple interdependent signaling pathways including those controlled by PKC and MAPK and calcium are involved in the stimulation of hypertrophy. The role of calcium in regulating hypertophy is, however, paradoxical since it is also responsible for the stimulation of myocyte contraction during every heart beat. How, calcium can control both myocyte contraction and transcription with such great specificity and fidelity is the focus of significant effort. One possibility is that changes in the amplitude and/or frequency of the calcium changes that occur within the myocyte during exercise or neurohumoral stimulation, acting through calcium sensitive kinases and phosphatases initiate the hypertrophic response. A further possibility is that the calcium signals that drive transcription and hypertrophy occur spatially or temporally independent of the bulk calcium changes that cause contraction. Recently, we have shown that inositol 1,4,5 trisphosphate ($InsP_3$) synthesised downstream of Gq-coupled plasma membrane receptors mobilises calcium from intracellular stores in cardiac myocytes, increasing the amplitude of the depolarisation-induced calcium transients and the frequency of spontaneous calcium release events. Moreover, $InsP_3Rs$ are expressed in cardiac myocytes and are localised to the cell periphery and to the nuclear membrane. We now show that $InsP_3$-induced calcium release can also specifically regulate the expression of hypertrophy-associated genes and increases in cell size. We also show that $InsP_3$-induced calcium release contributes to hypertrophy induced by activation of L-type voltage-operated channels (which increases the rate of contraction) and by β-adrenergic stimulation. High-speed confocal imaging reveals that $InsP_3$-induced calcium release occurs in a perinuclear region in neonatal cardiac myocytes. The spatial segregation of these $InsP_3$-induced calcium release events from the bulk changes in calcium that stimulates contraction ideally places them to specifically control transcription within the nucleus. Taken together, these data raises the possibility that $InsP_3$-induced calcium release is a focus of diverse pro-hypertrophic pathways and is a fundamental signaling mechanism in the cardiac myocyte.

Keywords: calmodulin, gene expression, cyclosporine, calcineurin, disease, cardiac hypertrophy

NAADP: FROM SECOND MESSENGER TO THERAPY

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Nicotinic acid adenine dinucleotide phosphate (NAADP) is a potent Ca^{2+}-mobilizing nucleotide involved in T cell Ca^{2+} signaling [1]. Stimulation of the T cell receptor/CD3 complex resulted in biphasic kinetics of NAADP formation, starting with a rapid peak (10 sec) and a sustained phase between 5 and 20 to 30 min [2]. Formation of NAADP was analysed using a novel enzymatic cycling assay with fmolar sensitivity [2].
In T-lymphocytes, microinjection of NAADP evoked local and global Ca^{2+} signals which depend on the functional expression of ryanodine receptors [3,4]. Using combined microinjection and high resolution confocal calcium imaging, we demonstrate that subcellular Ca^{2+} signals, characterized by amplitudes between ~30 and 100 nM and diameters of ~0.5 µm, preceded global Ca^{2+} signals. Co-injection of the ryanodine receptor antagonists ruthenium red and ryanodine together with NAADP abolished the effects of NAADP [3,4]. This pharmacological approach was confirmed by a molecular knock-down approach. Jurkat T cell clones with largely reduced expression of ryanodine receptors [5] did neither respond with local nor global Ca^{2+} signals to microinjections of NAADP [3,4].
Small molecular NAADP antagonists were synthesized and characterized. A lead NAADP antagonist blocked TCR/CD3 mediated Ca^{2+} signaling and activation of primary T cells *in vitro*. Moreover, a significant therapeutic effect was observed in transfer experimental autoimmune encephalomyelitis in Lewis rats, an established model for multiple sclerosis.

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Keywords: NAADP, ryanodine receptor, T cell, experimental autoimmune encephalomyelitis, InsP3, cardiac myocytes, cardiac hypertrophy, disease

A FRAP-BASED ANALYSIS OF THE ASSEMBLY AND DYNAMICS OF SARCOPLASMIC RETICULUM PROTEINS IN SKELETAL MUSCLE CELL

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The organization of specific domains of the sarcoplasmic reticulum, like terminal cisternae and longitudinal tubules, requires the selective redistribution of specific proteins in these domains. In undifferentiated myoblasts, most sarcoplasmic reticulum proteins appear to be diffused. Following differentiation, a clear redistribution of these proteins occurs whereby specific domains are formed. Domains enriched in ank1.5 can be observed starting at 2 days of differentiation. These ank1.5 domains are distinct from the junctional domains that contain triadic proteins, like triadin and RyRs, and from domains enriched in SERCA pumps. The timing and the efficiency of these proteins to localize to specific regions of the sarcoplasmic reticulum were found to differ, suggesting the existence of a regulated hierarchical process in the organization of the mature sarcoplasmic reticulum. To obtain more information on this process we generated Green Fluorescent Protein- (GFP) fusion proteins of ank1.5, triadin, and SERCA and the mobility of these proteins was analysed in FRAP experiments. FRAP-based experiments revealed that all these GFP-proteins were apparently free of moving in non-differentiated cells, while dramatic differences in their mobility were observed in differentiated cells. These different dynamics are strongly suggestive that specific retention mechanisms may be involved in the localization of SR proteins within specific sub-domains. Molecular and biochemical experiments are been performed to identify the molecules involved in the organization of these SR domains.

Keywords: sarcoplasmic reticulum, calcium release, muscle differentiation. GFP-fusion proteins.

RATIONAL DESIGN AND PREDICTION OF CALCIUM BINDING PROTEINS

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To overcome the limitations of solely investigating naturally occurring Ca^{2+}-binding proteins, we have developed designing and grafting approaches for creating a single Ca^{2+}-binding site in order to dissect the key structural factors that control Ca^{2+}-binding affinity, conformational change, cooperativity and dynamic properties. We have demonstrated the successful design of an array of single Ca^{2+}-binding sites with varying Ca^{2+}-binding affinities in the non-Ca^{2+}-binding cell adhesion protein, CD2 and green fluorescent protein (GFP). These designed Ca^{2+}-binding proteins retain their biological functions. The solution structure reveals that Ca^{2+} binds at the intended site with the designed arrangement, which validates our general strategy for designing and identifying Ca^{2+}-binding proteins. In addition, we have shown that charged ligand residues play a dominant role for calcium binding affinity, stability, and calcium-dependent global conformational change. Furthermore, we have applied grafting approach to obtain the site-specific metal binding affinity and to estimate cooperativity of continuous Ca^{2+}-binding sites using a scaffold protein. Moreover, we have developed several computer algorithms to identify calcium binding sites in proteins from either genomic information or the geometric properties of oxygen clusters coupled with the graph theory. We have further applied our grafting approach to verify the metal-binding capacity of several predicted Ca^{2+}-binding sites in several classes of proteins including Ca-sensing receptor, rubella virus nonstructural protease, and bacterial calcium binding proteins. The success of the approaches that we have developed confirms the efficacy of our computational methods for identifying Ca^{2+}-binding sites in proteins; and more significantly, it opens up an excellent opportunity for investigating the mechanism(s) underlying Ca^{2+}-modulated function.

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Key words: Calcium-binding proteins, protein design, grafting, calciomics

COMBINED FIXATIVE METHOD FOR THE CALCIUM-DEPENDENT CYTOSOLIC/MEMBRANE-BINDING PROTEINS: THE CASE OF ANNEXIN 2

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One of the most important techniques in cell biology is microscopy. In recent years the development fluorescent probes and GFP-derived chimaeras leads to a better understanding of molecular dynamics in living cells. However, it is not always possible to study the proteins in living cells. Sometimes GFP chimaeras do not behave like the endogenous proteins, and sometimes their over-expression induces a mislocalisation. In such cases, the traditional (however static) immunofluorescence techniques are of unavoidable high value. Cell fixation is not only crucial for the conservation of cell structures and protein epitopes, but also the fixation procedure must not change the distribution of proteins in the cell.

The annexin family of calcium- phospholipid-binding proteins is to a group of calcium dependent cytosolic-membrane proteins. Contradictory data in the literature concern the localisation of a particular annexins. These contradictions are sometimes due to the differences in the protocols used to visualize the distribution of the proteins (fixation and type of antibodies). Another important factor for annexin studies is that when bound to membranes, the epitopes could be protected from antibody. This is especially important for annexins that induce membrane aggregation (annexins 1, 2 and 7).

In our laboratory, we observed that the localisation of annexin 2 depends on the fixative used. We analysed the effects of different fixative methods on the distribution of annexin 2 in epithelial cells. We compared the localisation of annexin 2 after fixation in paraformaldehyde, methanol and mixed methods. We compared the distribution of an annexin 2-GFP chimaera in living cells with the distribution in fixed cells, and with the distribution of annexin 2 revealed by immunofluorescence. The results suggest that a mixed protocol consisting of paraformaldehyde and methanol fixation is the best to avoid artefactual redistribution of the protein and epitope masking.

Keywords: annexin 2, immunofluorescence, fixative, GFP Chimaeras

SLM PROTEINS ARE NOVEL YEAST CALCINEURIN SUBSTRATES MEDIATING THE CELLULAR RESPONSE TO HEAT STRESS

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The Ca^{2+}/calmodulin-dependent phosphatase, calcineurin, promotes yeast survival during environmental stress. We identified Slm1 and Slm2, two related PH-domain containing proteins that are redundant for an essential function, as novel calcineurin substrates required for the execution of sphingolipid-dependent processes during heat stress (1). Slm1 and Slm2 bind to calcineurin via docking sites that are required for their dephosphorylation by calcineurin, and are related to the PxIxIT motif identified in NF-AT. *In vivo*, calcineurin mediates prolonged dephosphorylation of Slm1 and Slm2 during heat stress, and this response can be mimicked by exogenous addition of the sphingoid base, phytosphingosine. Slm proteins also promote the growth of yeast cells in the presence of myriocin, an inhibitor of sphingolipid biosynthesis, and regulation of Slm proteins by calcineurin is required for their full activity under these conditions. In addition, Slm proteins function downstream of phytosphingosine, since cells lacking Slm activity are not rescued by exogenous phytosphingosine. During heat stress, sphingolipids signal turnover of the uracil permease, Fur4. In cells lacking Slm protein activity, stress induced endocytosis of Fur4 is blocked, and Fur4 accumulates at the cell surface in an ubiquitinated form. Furthermore, cells expressing a version of Slm2 that cannot be dephosphorylated by calcineurin display an increased rate of Fur4 turnover during heat stress. Thus, calcineurin may modulate sphingolipid-dependent events through regulation of Slm1 and Slm2. These findings, in combination with previous work identifying Slm1 and Slm2 as targets of Mss4/PIP_2 and TORC2 signaling (2), suggest that Slm proteins are at an important regulatory node integrating information from a variety of signaling pathways, including Ca^{2+}/calcineurin and sphingolipid signaling, to coordinate the cellular response to heat stress.

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Keywords: yeast, Ca^{2+} signaling, sphingolipids, calcineurin, heat stress

MODULATION OF CALCIUM SIGNALING BY DOMINANT NEGATIVE SPLICE VARIANT OF RYANODINE RECEPTOR SUBTYPE 3 IN NATIVE MYOCYTES.

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The ryanodine receptor subtype 3 (RYR3) is expressed ubiquitously but its physiological function varies from cell to cell. Here, we investigated the role of a dominant negative RYR3 isoform in Ca^{2+} signaling in native smooth muscle cells. We used intranuclear injection of antisense oligonucleotides to specifically inhibit endogenous RYR3 isoform expression. In mouse duodenum myocytes expressing RYR2 subtype and both spliced and non-spliced RYR3 isoforms, RYR2 and non-spliced RYR3 were activated by caffeine whereas the spliced RYR3 was not. Only RYR2 was responsible for the Ca^{2+}-induced Ca^{2+} release mechanism that amplified Ca^{2+} influx- or inositol 1,4,5-trisphosphate-induced Ca^{2+} signals. However, the spliced RYR3 negatively regulated RYR2 leading to the decrease of amplitude and upstoke velocity of Ca^{2+} signals. Immunostaining in injected cells showed that the spliced RYR3 was principally expressed near the plasma membrane whereas the non-spliced isoform was revealed around the nucleus. This study shows for the first time that the short isoform of RYR3 controls Ca^{2+} release through RYR2 in native smooth muscle cells.

Keywords: Ryanodine receptor, RyR2, RyR3, Splice variant, CICR.

BINDING OF IRBIT TO THE IP_3 RECEPTOR: DETERMINANTS AND FUNCTIONAL EFFECTS

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IRBIT has previously been shown to interact with the inositol 1,4,5-trisphosphate (IP_3) receptor (IP_3R) in an IP_3-sensitive way (Ando *et al.*, 2003). So far it remained to be elucidated whether this interaction was direct or indirect, and whether it was functionally relevant. We now show that IRBIT can directly interact with the IP_3R, and that both the suppressor domain and the IP_3-binding core of the IP_3R are essential for a strong interaction. Moreover, we identified a PEST motif and a PDZ-ligand on IRBIT which were critical for the interaction with the IP_3R. Furthermore, we identified Asp-73 as a critical residue for this interaction. Finally, we demonstrated that this interaction functionally affects the IP_3R: IRBIT inhibits both IP_3 binding and IP_3-induced Ca^{2+} release (Devogelaere *et al.*, 2006).

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Keywords: IRBIT, PEST motif, PDZ-ligand, inositol 1,4,5-trisphosphate receptor, IP_3-induced Ca^{2+} release, point-mutation

TOPOLOGY OF CELLULAR NETWORKS IN CALCIUM SIGNALING

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Mitochondria and cytosolic Calcium-binding proteins play an important role in the control of cellular metabolism by Calcium ions (CCa^{2+}). We propose a model of intracellular Ca^{2+} oscillations which includes Ca^{2+} fluxes through the plasma membrane of cell and the influence of mitochondria and cytosolic Ca^{2+}-binding proteins. In addition we include the dependence of Ca^{2+} fluxes through the membrane of the endoplasmic reticulum as a function of the level of inositol 1,4,5-trisphosphate (IP_3IP_3) in the cell.
We use this intracellular model to analyze the mechanisms of intercellular Ca^{2+} signaling through gap junctions between two cells. We also take into account the dependence of the conductivity of gap junctions on cytosolic Ca^{2+} concentration [1]. Our results allow us to conclude that the mechanism of transmission of Ca^{2+} signals from the stimulated cell to an adjacent cell is due to the diffusion of IP_3 through gap junctions, whereas the mechanism of synchronization of Ca^{2+} signals in the two cells is due to Ca^{2+} diffusion.
We use these results to analyze the influence of the topology of the connection graph of several (3 or 4) cells on intercellular Ca^{2+} wave propagation. Depending on the topology, both a fast decline of the intercellular Ca^{2+} wave and a steady expansion of the intercellular Ca^{2+} wave takes place. Moreover, for different configurations of intercellular connections in the same group of cells, different Ca^{2+} behavior is observed.
Furthermore, we generalize our research to the level of the tissue. We model Ca^{2+} wave propagation in airway epithelial cells, which are connected to each other by gap junctions. We model the complex structure of cellular connections in tissue by a delaunay triangulation and the corresponding voronoi diagram. Qualitative comparison with experiment [2] shows that this modeling of the network has an essential influence on the problem and allows us to obtain results which are not observed on the square net. Namely, heterogeneous Ca^{2+} wave propagation in different directions, many kinds of branches, fingers and so on, which are observed experimentally. Therefore we conclude that the topology of connections between cells an play an important role in alcium wave propagation in cells and tissue.

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Keywords: Calcium wave, mitochondria, gap junction, connectivity

THEORETICAL INVESTIGATION OF Ca^{2+}/CaM DEPENDENT MLCK ACTIVATION AND MYOSIN PHOSPHORYLATION/ DEPHOSPHORYLATION

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Recently we published the mathematical model of a detailed kinetic scheme describing interactions between Ca^{2+}, calmodulin (CaM) and myosin light chain kinase (MLCK). The model takes into account different binding states of CaM with respect to the N- and C-terminal binding sites for Ca^{2+} and the binding site for MLCK, yielding eight different aggregates [1]. The important result of the model is the prediction of Ca^{2+} dependent active form of MLCK which is in the model taken as proportional to the concentration of Ca_4CaM·MLCK complex. The result is in reasonable agreement with the first direct measurement of the amount of active MLCK versus Ca^{2+} [2]. It presents a significant progress in the modelling of these interactions as we pointed out in ref. [3]. Here we present the mathematical model of Ca^{2+}/CaM dependent MLCK activation in the presence of the substrate for MLCK, i.e. myosin, by taking into account phosphorylation and dephosphorylation of myosin modelled by M-M kinetics. This approach in modelling enables the prediction of MLCK activity and, thus, comparison to a much broader set of data for MLCK activity and myosin phosphorylation measured by different authors. Furthermore, the sensitivity of the main system variable, i.e. myosin phosphorylation, is studied with the control theory. The emphasis is given on the influence of elevated total concentration of MLCK; the property of bronchial muscle cells obtained from asthmatic subjects [4]. We can show by the model that elevated MLCK affects the myosin phosphorylation and, hence, the magnitude of force. Moreover, our complex model for Ca^{2+}/CaM dependent MLCK activation predicts that the process of MLCK activation is much slower as expected and predicted by the earlier models. This characteristic is especially evident in the range of physiologically significant low Ca^{2+} concentrations. This is an inherent dynamical property of the system and it is discussed in the view of relatively slow temporal evolution of force and the time delay of force with respect to Ca^{2+} transient in smooth muscle cells that consequently lead to stationary value of force on the cellular level even in the case when Ca^{2+} oscillates.

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Keywords: calcium, calmodulin, MLCK, activity, myosin, phosphorylation

RYR1 KO MOUSE REVEALS A FUNCTIONAL FKBP REGULATION OF RYR2 IN EMBRYONIC URINARY BLADDER MYOCYTES

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Ryanodine receptors (RYR) are intracellular channels that regulate excitation-contraction coupling and calcium homeostasis in smooth muscle. Local calcium signals such as calcium sparks and calcium-induced calcium release (CICR)-activated calcium waves rely on RYR activation. However, the role of each RYR subtypes, as well as the role of the stabilizing RYR associated members of the FK506 Binding Protein (FKBP) family in calcium signaling remains controversial in smooth muscle. In adult mouse urinary bladder myocytes, the crucial importance of RYR2 has been indirectly demonstrated [1]

In order to determine the role of the RYR1 subtype in RYR-associated calcium signals in embryonic urinary bladder myocytes, we compared these signals in myocytes from wild-type and RYR1 KO mouse [2]. Local calcium signals could be observed in RYR1 KO myocytes, without differences in their parameters with those observed in wild-type myocytes. Depolarisation- and caffeine-induced calcium waves could be activated with a significant decrease in amplitude in KO myocytes.
An antibody based strategy confirmed that in RYR1 KO myocytes, these calcium signals were particularly dependent on the activation of the RYR2 subtype. We sought to determine if adaptative mechanisms could have been set in RYR1 KO mouse urinary bladder to counteract the lack of expression of RYR1. In RYR1 KO embryonic urinary bladder, RT-PCR and western-blots studies indicated no overexpression of either RYR2 or RYR3, but a diminution in the expression of FKBP. This was correlated with an absence of regulation of the caffeine-induced calcium response by the FKBP-RYR uncoupler rapamycin in KO myocytes compared to wild-type myocytes.

These results formally demonstrate that in RYR1 KO embryonic urinary bladder myocytes, RYR-associated calcium signaling is dependent on a FKBP-non stabilized RYR2. They also suggest a role for RYR1 in wild-type myocytes, when RYR2 is in a stabilized state.

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Keywords: Calcium sparks, calcium waves, RYR1 KO mouse, confocal microscopy, urinary bladder myocytes.

INOSITOL 1,4,5-TRISPHOSPHATE ($InsP_3$)–INDUCED CALCIUM RELEASE CONTROLS CARDIAC MYOCYTE HYPERTROPHY

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As a result of increased haemodynamic load, genetic factors or disease, the heart undergoes a hypertrophic response. Genetic manipulation of components of various Ca^{2+} regulated gene transcription pathways has revealed that this ion has a fundamental role in stimulating cardiac hypertrophy. However, it is difficult to reconcile how Ca^{2+} sensitive gene transcription can be controlled with great fidelity and specificity independent of the increases in Ca^{2+} that occur during every heart beat. Previously, we have shown that Endothelin (ET-1) stimulates $InsP_3$-dependent Ca^{2+} release in atrial and ventricular myocytes (1). ET-1 is also a potent inducer of cardiac hypertrophy. Here we investigated whether ET-1/$InsP_3$- induced Ca^{2+} release contributed to its pro-hypertrophic action.

Experiments were performed using spontaneously contracting monolayers of primary cultures of neonatal ventricular rat myocytes (NRVM). NRVMs were prepared by collagenase digestion of cardiac ventricles isolated from 1-2 day old Wistar rat pups. Quantitation of atrial natruiretic factor (ANF) by immunofluorescence and real-time PCR, together with cell surface area were used as measures of hypertrophy.

To investigate the mechanism underlying ET-1 induced hypertrophy, 2-APB (10 μM) was first used to inhibit $InsP_3$-induced Ca^{2+} release. 2-APB significantly decreased the percentage of myocytes expressing ANF following ET-1 stimulation (100 nM, 24 hr). To further demonstrate the $InsP_3$-dependence of this process, $InsP_3$-induced Ca^{2+} release was inhibited by adenoviral mediated expression of $InsP_3$- 5'phoshatase. In myocytes expressing 5'phosphatase, ET-1-induced ANF expression was significantly inhibited when compared to ET-1-stimulated GFP expressing cells. To determine whether Ca^{2+} entry arising through voltage gated channels was required for ET-1-induced hypertrophy, ET-1 was applied to cells in which voltage gated channels were blocked with nifedipine (10 μM) and mibefridil (1.8 μM). The percentage of cells expressing ANF was significantly lower in these inhibited cells in comparison to controls. Under these conditions, ET-1 significantly increased the percentage of ANF expressing cells.

Together, these data demonstrate that $InsP_3$-induced Ca^{2+} release is required for the induction of hypertrophy as a result of Gq stimulation or increased workload. Furthermore, this provides a mechanism by which gene transcription can be isolated from the increases in cytosolic Ca^{2+} that occur during every heart-beat.

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Keywords: Signaling, calcium, inositol 1,4,5-trisphosphate, hypertrophy, heart failure

INHIBITION OF INOSITOL 1,4,5-TRISPHOSPHATE ($INSP_3$)-INDUCED CALCIUM RELEASE (IICR) BY THE CALCIUM BINDING PROTEIN CALMYRIN

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Inositol 1,4,5-trisphosphate receptors ($InsP_3Rs$) are widely-expressed intracellular channels that release Ca^{2+} from internal stores in response to many physiological stimuli. It is becoming apparent that $InsP_3Rs$ form complexes with a multitude of accessory proteins. Many of the proteins that bind to $InsP_3Rs$ appear to modulate channel function and Ca^{2+} release.
$InsP_3Rs$ are known to be regulated by the ubiquitous EF-hand Ca^{2+}-binding protein calmodulin (CaM). We recently demonstrated that members of the neuronal 'calcium binding protein' family (CaBPs), which also bind Ca^{2+} using EF-hand motifs and have approximately 50% homology with CaM, functionally interact with $InsP_3$ receptors ($InsP_3R$) and inhibit IICR (1). In the present study, we examined the putative interaction of another EF-hand-containing protein, calmyrin, which is ubiquitously expressed and has 56% homology to CaM.

We found that calmyrin interacts with the NH_2-terminus of $InsP_3Rs$ in a Ca^{2+}-independent manner. Video imaging of fura-2 loaded COS7 cells over-expressing YFP-tagged calmyrin revealed that calmyrin inhibited Ca^{2+} release from internal stores induced by purinergic agonist (ATP; 0.5, 1 and 100 μM). A mutated protein in which the consensus site for myristoylation was altered ('calmyrin-G2A') also inhibited IICR. Unlike wild-type calmyrin, which was largely bound to cellular membranes, the calmyrin-G2A mutant was diffuse. From these data we concluded that calmyrin function is not dependent on its myristoylation and hence membrane targeting. Recombinant calmyrin also inhibited $InsP_3$-dependent $^{45}Ca^{2+}$ flux from permeabilised cells. The calmyrin-mediated inhibition of Ca^{2+} release was $InsP_3R$ specific since there was no inhibition of caffeine-induced Ca^{2+} release from ryanodine receptors. To investigate the mechanism by which calmyrin mediates its effect we performed an $InsP_3$ binding assay using recombinant $InsP_3R$ ligand binding domain. In these studies we demonstrated that calmyrin inhibited $InsP_3$ binding to the $InsP_3R$ in a calcium-independent manner.

Our data indicate that calmyrin interacts with $InsP_3Rs$ and inhibits IICR in a similar manner to CaBPs and CaM.

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MODELING CALMODULIN STRUCTURE, DYNAMICS AND INTERACTIONS WITH TARGETS

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Calmodulin (CaM) is a small protein involved in calcium signaling. Calcium binding induces a a structural change in CaM, modifying its interactions with targets, resulting in a modulation of the activity of target proteins. We present results of molecular dynamics simulations of nanosecond motions for calmodulin in its three functional forms: calcium-free, calcium-loaded, and in complex with both calcium and a target peptide, a fragment of the smooth muscle mysosin light chain kinase (smMLCK) [1]. The most interesting information provided by the simulations is that the dynamics of calcium-loaded and calcium-free calmodulin in solution is dominated by slow rigid motions of the globular N-terminal and C-terminal domains. In these motions the interdomain distance varies by up to 10 Å, and the relative orientations of the domains change by up to 110^{o}. The large flexibility is in accord with NMR and fluorescence experiments. Our simulations support the standard model of CaM activation, in which calcium binding induces exposure to solvent of hydrophobic patches, which become primed for interaction with targets. However, to bring simulations and experiments into accord, we propose a redefinition of the hydrophobic patches as those non-polar residues which are actually involved in target binding in the CaM:smMLCK peptide complex. In contrast to the calcium-free and calcium-loaded systems, the calmodulin-peptide complex was quite rigid and did not exhibit any significant domain motions. Using a Poisson-Boltzmann model for electrostatic interactions with a cavity term proportional to the solvent exposed surface area, we calculated an interaction free energy of -100 kcal/mol for the CaM:peptide complex. The contributions were –64 kcal/mol from buried surface area and –36 kcal/mol from electrostatics, consistent with dominance of the hydrophobic effect. In the nonpolar interactions a crucial role is played by the nine methionines of calmodulin. The experimental binding free energy is about -10 kcal/mol. The difference between this value and the calculations may be explained by the large conformational entropy loss due to complexation and loss of structural flexibility of CaM and peptide, which we estimate at 80-90 kcal/mol. We also report our preliminary studies on a semi-empirical approach to predict calmodulin-target binding affinities based on structures of known complexes, published thermodynamic data and simplified simulations. Overall, our simulation results were consistent with a wide range of experimental studies, including NMR, fluorescence, x-ray, cross-linking, calorimetry and mutagenesis. Additionally, a range of mechanistic microscopic information emerged which is useful in explaining the activity of the protein but is difficult to measure experimentally.

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Keywords: calmodulin, molecular dynamics simulations, domain motions, calmodulin-peptide interactions

IS MELATONIN EFFECT ON $[Ca^{2+}]_i$ IN CHICKEN AND MOUSE SPLENOCYTES MEDIATED VIA G-PROTEIN COUPLED SPECIFIC MELATONIN RECEPTORS?

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Among numerous physiological functions attributed to the pineal hormone, melatonin (Mel), its role in immunomodulation is well established. Mel was found to modify immunity in different species but the mechanism(s) of its action are still poorly understood. One of the proposed mechanisms involves a mediation of specific membrane bound G-protein coupled Mel receptors present, among others, in mouse and chicken splenocytes. On-going research shows a complicated circadian regulation of expression and/or function of Mel receptors. Proliferation, a good measure of immune cells activity, is associated with the changes in intracellular Ca^{2+} level ($[Ca^{2+}]_i$). It has been demonstrated that in vitro Mel effect on splenocyte proliferation is mediated through its specific membrane receptors. The aim of present study was therefore to examine: 1) the in vitro effect of Mel on the $[Ca^{2+}]_i$ in chicken and mouse splenocytes isolated at mid-day and mid-night of 12 h-photoperiod; 2) involvement of Mel membrane receptors in this effect. Isolated lymphocytes were loaded with Fura-2AM, treated with Mel receptor agonists, Mel or 2-Iodomelatonin, and Mel receptors antagonist, luzindole. $[Ca^{2+}]_i$ was measured in cell suspension using RF-5000 recording spectrofluorimeter in Ca^{2+}-containing and Ca^{2+}-free medium.
Melatonin increased $[Ca^{2+}]_i$ in chicken and mouse splenocytes isolated during the day and at night, respectively. This increase was unaffected by co-incubation with luzindole. 2-iodomelatonin stimulated $[Ca^{2+}]_i$ regardless the time of day of cell isolation. In chicken splenocytes kept in Ca^{2+}-free medium the effect of Mel was diminished, while the effect of 2-iodomelatonin remained the same comparing to the Ca^{2+}-containing medium. On the contrary, in mouse splenocytes lack of Ca^{2+} in the medium reduced the effect of both Mel receptor agonists.
Results described herein suggest that melatonin differently affected $[Ca^{2+}]_i$ in mouse and chicken splenocytes. 2-iodomelatonin binds to Mel receptors with higher affinity than Mel, therefore its effect does not depend on the time of day. Melatonin receptors seem to be desensitised at night in chicken and during the day in the mouse splenocytes. Moreover, in mouse lymphocytes the effect of Mel receptor agonists involve a mobilisation of Ca^{2+} from intracellular stores as well as a capacitative calcium entry. On the other hand, in chicken splenocytes mechanism of Mel action seems to be less dependent on the presence of extracellular calcium. The precise role of melatonin receptors in generation of $[Ca^{2+}]_i$ changes remains to be elucidated.

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Keywords: melatonin, melatonin receptors, luzindole, splenocytes, chicken, mouse

Ca^{2+} RELEASING MESSENGERS DETERMINE AGONIST-SPECIFIC RESPONSE BY CONTROLLING THE BALANCE BETWEEN INTRACELLULAR CA^{2+} STORES

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Pancreatic acinar cells possess an apical secretory pole containing thin ER extensions, zymogen granules and lysosomes, but their respective contribution to agonist-evoked local Ca^{2+} spikes is not well understood. In these cells, acetylcholine (ACh), cholecystokinin (CCK) and the messengers cyclic ADP-ribose (cADPR), nicotinic acid adenine dinucleotide phosphate (NAADP), and inositol 1,4,5-trisphosphate (IP_3) evoke repetitive local Ca^{2+} spikes in the apical pole. Our work reveals that secretagogue-evoked local Ca^{2+} spikes require interaction of acid Ca^{2+} stores and the endoplasmic reticulum (ER) but in different proportions. CCK and ACh recruit Ca^{2+} from lysosomes and from zymogen granules through different mechanisms, via NAADP and cADPR respectively for CCK and via Ca^{2+} and IP_3, respectively for ACh. In addition, NAADP recruits Ca^{2+} from another acidic source and, to a lesser extent, from the ER. We also found that cADPR-evoked repetitive local Ca^{2+} spikes are more dependent on the ER than IP_3-evoked local spikes. We propose that multiple Ca^{2+} releasing messengers determine specific agonist-elicited Ca^{2+} signatures by controlling the balance between different acidic Ca^{2+} stores and the ER.

Keywords: lysosomes /Acetylcholine/Cholecystokinin/cADPR/NAADP/IP_3

ACETYLCHOLINE ACTIVATES RYR2-DEPENDANT CALCIUM OSCILLATIONS VIA CADPR PRODUCTION.

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In this study, we characterized the signaling pathway activated by acetylcholine that encodes Ca^{2+} oscillations in rat duodenum myocytes. These oscillations were observed in intact myocytes after removal of external Ca^{2+}, in permeabilized cells after abolition of the membrane potential and in the presence of heparin (an inhibitor of inositol 1,4,5-trisphosphate receptors) but were inhibited by ryanodine indicating that they were dependent on Ca^{2+} release from intracellular stores through ryanodine receptors. Ca^{2+} oscillations were selectively inhibited by methoctramine (a M2 muscarinic receptor antagonist). The M2 muscarinic receptor-activated Ca^{2+} oscillations were inhibited by 8-bromo cyclic adenosine diphosphoribose and inhibitors of adenosine diphosphoribosyl cyclase ($ZnCl_2$ and anti-CD38 antibody). Stimulation of ADP-ribosyl cyclase activity by acetylcholine was evaluated in permeabilized cells by measuring the production of cyclic guanosine diphosphoribose (a fluorescent compound) which resulted from the cyclization of nicotinamide guanine dinucleotide. As duodenum myocytes expressed the three subtypes of ryanodine receptors, an antisense strategy revealed that the ryanodine receptor subtype 2 alone was required to initiate the Ca^{2+} oscillations induced by acetylcholine but also by cyclic adenosine diphosphoribose and rapamycin (a compound that induced a uncoupling between 12/12.6 kDa FK506-binding proteins and ryanodine receptors). Inhibition of cyclic adenosine diphosphoribose-induced Ca^{2+} oscillations after rapamycin treatment confirmed that both compounds interacted with the ryanodine receptor subtype 2. Our findings show for the first time that the M2 muscarinic receptor activation triggered Ca^{2+} oscillations in duodenum myocytes by activation of the cyclic adenosine diphosphoribose / FK506-binding protein / ryanodine receptor subtype 2 signaling pathway.

Keywords: cyclic ADP ribose, ryanodine receptors, Ca^{2+} oscillations, acetylcholine, smooth muscle

SNAP-25 IS A NOVEL INOSITOL 1,4,5-TRISPHOSPHATE RECEPTOR ($InsP_3R$) INTERACTING PROTEIN THAT REGULATES INTRACELLULAR Ca^{2+} RELEASE AND INFLUX

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Inositol 1,4,5-trisphosphate receptors ($InsP_3Rs$) are major intracellular Ca^{2+} release channels in non-excitable cells. In addition to regulation by $InsP_3$ and Ca^{2+}, they are modulated by post-translational modification and the binding of accessory proteins. Thus, $InsP_3Rs$ act as scaffolds, anchoring many signaling proteins to the site of Ca^{2+} release, where they either regulate, or are regulated by Ca^{2+} release through the channel (1). To identify novel $InsP_3R$ interacting proteins, a proteomic approach was used. To this end, $InsP_3Rs$ were immunoprecipitated from rat brain lysate, which is a rich source of type 1 $InsP_3Rs$. Co-immunoprecipitated proteins were subsequently eluted and subjected to 2 dimensional liquid chromatography and Matrix Assisted Laser Desorption Ionization Time-of-flight Mass Spectrometry analysis (MALDI-TOF). Using this strategy, we identified the SNARE-associated protein SNAP-25. This interaction was confirmed by subsequent immunoblotting of proteins co-immunoprecipitated with the $InsP_3R$. SNAP-25 is a protein involved in Ca^{2+}-regulated secretory processes and associates with N- and P/Q-type voltage-operated plasma membrane Ca^{2+}-channels.

To test whether the expression of SNAP-25 affects Ca^{2+}-signaling, we established stable HeLa cell lines that expressed YFP-SNAP-25 in a tetracycline-inducible manner. In these cells, YFP-SNAP-25 was targeted to the plasma membrane. Using fura-2 imaging, we investigated whether overexpression of YFP-SNAP-25 affected $InsP_3$-induced Ca^{2+}-release and store operated Ca^{2+}-entry. Cells overexpressing YFP were used as control.
To investigate the effect of SNAP-25 on $InsP_3$-induced Ca^{2+}-release, cells were stimulated with increasing doses of the $InsP_3$-generating agonist histamine (0.5, 1 and 100 µM). Cells overexpressing YFP-SNAP-25 exhibited significantly decreased Ca^{2+}-responses to all concentrations of histamine applied ($p<0.01$ for all three concentrations).
The rate of quench of cellular fura-2 fluorescence by Mn^{2+}, which was used as Ca^{2+} surrogate in these experiments, was used to measure the effects of SNAP-25 on Ca^{2+}-entry. In the YFP-SNAP-25 overexpressing cells, basal and histamine-induced Ca^{2+}-entry was significantly greater than in YFP-expressing control cells. The SNAP-25 enhanced Ca^{2+}-entry was inhibited by 1 µM Gd^{3+}, indicating that it occurred via the store operated Ca^{2+}-entry (SOCE) pathway. In summary, we have shown that SNAP-25 is a novel $InsP_3R$ interacting protein, which affects both, Ca^{2+}-release from the ER and SOCE.

Reference:
Roderick H.L. & Bootman, M.D. (2003): Bi-directional signaling from the $InsP_3$ receptor: regulation by calcium and accessory factors. Biochem Soc Trans 31, 950-953.

Keywords: $InsP_3$ receptor, SNAP-25, interacting proteins, SOCE

NON-INVASIVE *IN VIVO* IMAGING OF Ca^{2+} SIGNALING IN LIVE ANIMALS

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A major challenge in biology is to monitor signal transduction in a *bona fide* physiological context, *i.e.* in live, unrestrained and un-anaesthetized animals. We have developed a new technique based on whole animal bioluminescence imaging, where changes in local calcium concentrations [Ca^{2+}] can be non-invasively recorded in live animals. Transgenic mice expressing the mitochondrially targeted Ca^{2+} sensitive bioluminescent probe, GFP-aequorin, are imaged using a highly sensitive photon counting system for whole animals. With this approach, mitochondrial [Ca^{2+}] can be repeatedly detected during muscle contraction induced by motor-nerve stimulation over hours and with a high time resolution. Dynamic patterns of activity, representing localised and transient increases in [Ca^{2+}] can also be visualised in the intact animal during epileptic seizures. Furthermore, increases in [Ca^{2+}] can even be followed in freely moving animals. Non-invasive detection of Ca^{2+} signaling in the living animal offers a unique way to study un-anaesthetised and un-restrained animals in their physiological environment.

REGULATION OF INOSITOL 1,4,5-TRISPHOSPHATE RECEPTORS ($InsP_3R$) BY PROTEIN KINASE B

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$InsP_3$-induced Ca^{2+} signals regulate many cellular processes from fertilisation to cell death. In addition to regulation by $InsP_3$ and Ca^{2+}, $InsP_3R$ activity is modulated by protein-protein interactions and post-translational modifications. Using the Scansite motif-searching algorithm to interrogate the $InsP_3R1$ sequence for consensus sites for protein phosphorylation, we have identified a consensus site for phosphorylation by protein kinase B (PKB/Akt) at its COOH-terminus. PKB is a serine/threonine kinase that is recruited to the plasma membrane upon growth factor/agonist stimulation where it subsequently activated. A number of substrates for PKB have been identified, many of which are important in cell proliferation, cell survival and glucose metabolism. Here, we investigated whether the $InsP_3R$ is a bona-fide substrate for PKB and whether PKB affects $InsP_3$-mediated calcium signals.
To test whether $InsP_3Rs$ were phosphorylated by PKB, $InsP_3Rs$ were immunoprecipitated from HeLa cells that that had been maintained in ^{32}P orthophosphate and in which PKB activity had been modified using pharmacological or molecular approaches. Stimulation of serum-starved HeLa cells with either insulin (1 µg/ml, 5 mins) or FBS (10% v/v) resulted in an LY294002 (10 µM) sensitive phosphorylation of $InsP_3Rs$ (n=3). No phosphorylation was detected in non-stimulated cells. Furthermore, $InsP_3Rs$ isolated from HeLa cells grown under serum replete conditions were also phosphorylated in an LY294002-sensitive manner (n=3). By immunoprecipitation of overexpressed $InsP_3R1$, in which the candidate phosphorylatable serine was mutated to an alanine, we were able to determine that the predicted PKB consensus site was susceptible to phosphorylation by PKB.
The effect of $InsP_3R$ phosphorylation upon $InsP_3$-induced Ca^{2+} release was next investigated in HeLa stable cell lines inducibly overexpressing either constitutively active (CA)-PKB, kinase dead (KD)-PKB or YFP. Experiments were performed on three separate days on three cover slips per day. Statistical significance was determined by ANOVA. Using fura-2 imaging, we found that the percentage of cells responding to 0.5 µM histamine (4.01±2.7%, $p<0.05$) was significantly inhibited by PKB overexpression when compared to cells expressing KD-PKB (42.71±3.73) or YFP (54.18±8.77). This effect was due to a decrease in $InsP_3R$ sensitivity, since the amplitude of the Ca^{2+} signal induced by a cell permeant $InsP_3$-ester were also significantly inhibited by PKB overexpression (404.7±25.54 nM in controls vs 278.3±15.4 nM in CA-PKB expressing cells, $p<0.05$).
Taken together, we have shown a novel role for PKB in regulating calcium signaling via a direct interaction with the $InsP_3R$. We propose that this interaction decreases $InsP_3R$ sensitivity to agonist stimulation and creates a link between growth factor signaling and the $InsP_3R$/calcium-signaling cascade.

Keywords: Calcium imaging, bioluminescent probes, GFP.

REGULATION OF INOSITOL 1,4,5-TRISPHOSPHATE-INDUCED Ca^{2+} RELEASE BY PROTEIN KINASE B

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The inositol 1,4,5-trisphosphate (IP_3) receptor (IP_3R) is an intracellular Ca^{2+} release channel involved in intracellular Ca^{2+} signaling. The regulation of Ca^{2+} release through IP_3Rs is controlled by Ca^{2+}, IP_3, ATP, calmodulin, kinases/phosphatases and other interacting proteins.
By using an "in silico" approach, we have identified several consensus sequences for protein kinase B (PKB) mediated phosphorylation in the three IP_3R isoforms namely S2681 of IP_3R1, S2633 of IP_3R2 and both S1832 and S2608 of IP_3R3.
To investigate the functional effect of PKB-mediated IP_3R phosphorylation on IP_3-induced calcium release (IICR), "phosphomimetic" and "non-phosphorylatable" mutations were made in the potentially important phosphorylation sites of both IP_3R1 (S2681D, S2681A) and IP_3R3 (S1832D, S1832A, S2608D and S2608A). Furthermore, IICR was investigated following stable expression of those mutant proteins in DT-40 triple knock-out (DT-40 TKO) cells devoid of endogenous IP_3Rs. Ca^{2+} release was measured by fluorescence measurements on permeabilised cells, using fluo-3 as Ca^{2+} probe.
We found that both IP_3R1 and IP_3R3 are *in vitro* phosphorylated by PKB (PKBα/Akt1). Mutation of S2681 into a non-phosphorylatable alanine completely abolished the PKB-mediated phosphorylation of the C-terminal tail of IP_3R1. In cells expressing the S2681D IP_3R1, IICR was markedly enhanced compared to the non-phosphorylatable S2681A mutant. E.g. Ca^{2+} release induced by 100 nM IP_3 increased approximately ~3 fold in the S2681D IP_3R1 cell line compared to the S2681A IP_3R1 cell line even though their expression levels in DT-40 TKO cells were identical. In the cells expressing wild type (wt) IP_3R1, an intermediate Ca^{2+} release was observed, suggesting that the wt IP_3R1 could already be partially phosphorylated on this site. The higher sensitivity of the S2681D mutant is not due to a higher content of the Ca^{2+} stores since the thapsigargin-sensitive Ca^{2+} store of the S2681D cell line even had a lower Ca^{2+} content than those of the S2681A cell line.
Current analysis is focused on the investigation whether the enhanced sensitivity of the S2681D IP_3R1 is due to a higher IP_3 affinity and how this enhanced sensitivity relates to the anti-apoptotic action of PKB.
In conclusion, our results support a crucial role for PKB-mediated phosphorylation of IP_3R1 at S2681 in the regulation of IICR.

Keywords: IP_3 receptor, protein kinase B, calcium release, InsP3 receptor, PKB, apoptosis, phosphorylation.

TRUNCATION OF IP_3R'S BY CASPASE-3 AND CALPAIN: EFFECTS ON CALCIUM SIGNALING AND APOPTOTIC CELL DEATH

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Previous studies have shown that the inositol 1,4,5-trisphosphate receptor type 1 (IP_3R1) is cleaved by caspase-3 during apoptosis with the formation of a so-called C-terminal "channel-only domain" [a]. We've investigated both the properties of this domain regarding the Ca^{2+} signaling, and its role in apoptotic cell death.
IP_3R deficient B-lymphocytes (IP_3R-KO) expressing the channel-only domain, which are more susceptible to undergo apotosis, show a bigger thapsigargin induced Ca^{2+} leak from internal stores as compared to IP_3R-KO cells expressing the full size IP_3R1 as well as to IP_3R-KO cells expressing an inactive channel-only domain ("channel death domain").
$^{45}[Ca2^{+}]$ flux experiments and aequorin measurements in mouse L-fibroblasts with high IP_3R1-levels confirm the model of the formation of a leaky channel-only domain in the ER during the process of apoptosis.
Other data suggest that the presence of a leaky IP_3R can also play a role in pathological conditions. In mouse embryonic fibroblasts deficient in both presenilin genes, which have a higher basal level of apoptosis as compared to wild type cells, there is an upregulation of IP_3R1 with a concomittant higher expression level of the channel-only domain [b]. The truncated IP_3R1 fragment could also be observed in ageing mouse eggs, in agreement with an aberrant Ca^{2+} signaling observed.
As for the IP_3R type 3, we've observed similar results, i.e., the receptor is also cleaved during apoptosis, although this IP_3R isoform does not contain a caspase-3 cleavage motif (DEVD). We show that both *in vivo* and *in vitro* this cleavage is mediated by calpain. Moreover, the sensitivity to apoptotic stimuli can be reduced by incubation with calpain inhibitors. Furthermore we determined the exact calpain-cleavage site.
These data suggest that IP_3R1, and possibly also IP_3R3, play a pivotal role in the execution of apoptosis and this through the proteolytic formation of a leaky C-terminal channel-only domain.

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Keywords: Inositol 1,4,5–trisphosphate receptor, caspase-3, calpain, calcium leak, apoptosis

MONITORING NUCLEAR CALCIUM SIGNALS USING RECOMBINANT CALCIUM PROBES IN DROSOPHILA LARVAL BRAIN

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The generation of calcium signals following synaptic activity is a fundamental property of neurons that controls many neuronal processes including cell survival, learning and memory. In cultured neurons, increases in nuclear calcium concentrations are known to be critical for the activation of gene expression mediated by the transcription factor CREB (1). CREB has been implicated in transcription-dependent plasticity (late phases of LTP).
Confirming the importance of nuclear calcium signaling in the intact brain is impeded by the restrictions and complexities of in vivo experimentation. However, using genetically encoded Ca^{2+} indicators in the *Drosophila* central nervous system provides an excellent system to explore the role Ca^{2+} in an intact behaving animal. Since we are particularly interested in Ca^{2+} signaling of the nucleus, we are engineering genetically encoded nuclear Ca^{2+} indicators.
To visualize nuclear calcium signals in *Drosophila,* we have engineered a transgenic fly based on the GCaMP 1.3 (2), which increases its fluorescence when binding Ca^{2+}. We made several different GCaMP constructs with nuclear localization signals (NLS) and used the Gal4/UAS-system to direct the expression of the indicators to cells of interest (3). Because the larval neuro-muscular junction has been a simple and useful system to study plasticity, we first expressed these indicators in muscle and motorneurons of third-instar larvae. In both cell types, the Ca^{2+} indicator localized to the nucleus. In addition, we have tested both the standard GCaMP (4), and the NLS-GCaMP for detecting calcium transients in the larval brain in response to KCl-induced membrane depolarization.

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Keywords: CREB, LTP, calcium transients;

NOVEL ROLE OF NAADP IN THE REGULATION OF CARDIAC EXCITATION-CONTRACTION COUPLING

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A novel Ca^{2+} releasing messenger, nicotinic acid adenine dinucleotide phosphate (NAADP) mobilises Ca^{2+} from intracellular Ca^{2+} stores in various cell types [1]. Cardiac tissue expresses high affinity binding sites for NAADP [2], but there have been no previous functional studies of NAADP actions in intact cardiac cells.

Here we report that NAADP can increase Ca^{2+} transients in isolated guinea-pig ventricular myocytes. The rises in Ca^{2+} accompanying the action potential (Ca^{2+} transients) were compared before and after photolysis of NAADP using laser scanning confocal microscopy. Photolysis of caged-NAADP (5 μM) caused a progressive increase in Ca^{2+} transients with time that was greater at 60 seconds than at 20 seconds after photolysis. When the concentration of caged-NAADP was reduced from 5 to 1.5 μM, there were smaller but still significant increases in the amplitude of the Ca^{2+} transient following photolysis of caged-NAADP (41 ± 10 % at 5 μM, $n = 8$, $p < 0.05$, 19 ± 5 % at 1.5 μM, $n = 4$, $p < 0.05$). However, significant changes in Ca^{2+} transient were no longer detected at concentration of 0.5 μM. The effects were prevented by bafilomycin A1, a H^+-ATPase inhibitor, by desensitising concentrations of NAADP (1 mM) and by inhibition of the sarcoplasmic reticulum (SR) function with ryanodine and thapsigargin. The specific action of balilomycin A1 on acidic compartment, but not the SR, was confirmed by using Lysotracker Red, Bodipy FL ryanodine and Bodipy TR thapsigargin.

We propose that NAADP provokes Ca^{2+} release from an acidic Ca^{2+} store, which in turn enhances Ca^{2+} release from the SR by increasing the SR Ca^{2+} load. The proposal that NAADP plays a physiological role in cardiac muscle is strongly supported by the observation that β-adrenoceptor stimulation regulates NAADP production. When guinea-pig hearts were perfused with isoprenaline on a Langendorff apparatus, the NAADP level was found to be significantly increased from the resting level 0.220 ± 0.027 nmol/mg protein ($n = 10$) to 0.337 ± 0.43 nmol/mg protein ($n = 9$).

This study presents the first direct evidence that NAADP is a regulator of cardiac contractility.

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Keywords: NAADP, Ca transients

MODULATION OF LOCALIZED CYTOSOLIC CALCIUM OSCILLATIONS IN PANCREATIC ACINAR CELLS BY CALCIUM DIFFUSION RATE IN THE ENDOPLASMIC RETICULUM

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Cytosolic Ca^{2+} ($[Ca^{2+}]_c$) signals in non-excitable cells are primarily generated by release of the ion from the endoplasmic reticulum (ER). In physiological conditions, ER has a continuous network structure that allows diffusion of molecules, including Ca^{2+}, in the lumen. A gradient of $[Ca^{2+}]_{ER}$ caused by a localised Ca^{2+} release or Ca^{2+} uptake will facilitate intra-ER Ca^{2+} diffusion which will subsequently alter $[Ca^{2+}]_{ER}$. Because the rates of Ca^{2+} release and Ca^{2+} uptake are known to be highly sensitive to $[Ca^{2+}]_{ER}$, it has been suggested that intra-ER Ca^{2+} diffusion may be one of the major players in the regulation of the localised cytosolic Ca^{2+} signals.
In pancreatic acinar cells, localised $[Ca^{2+}]_c$ oscillations can be generated in the apical (secretory) part of the cell by a low concentration of a Ca^{2+}-releasing agonist or by direct intracellular application of a second messenger for Ca^{2+} release channels (e.g. $InsP_3$, cADPR, NAADP). The ER lumen in the apical part of the acinar cell is connected fully with the lumen of the basal ER and Ca^{2+} in the apical ER can therefore be replenished by Ca^{2+} entering through the basal plasma membrane. However, it is still not clear how intra-ER Ca^{2+} diffusion during localised Ca^{2+} oscillations in the apical region would affect the kinetics of the oscillations, such as frequency and amplitude of the oscillations, and their sensitivity to agonist concentration.
In this study, we investigated the relationship between the kinetics of the localised $[Ca^{2+}]_c$ oscillations and the rate of intra-ER Ca^{2+} diffusion using two-dimensional computational models of acinar cells and compared the properties of localised Ca^{2+} oscillations in the models with corresponding experimental data. First we monitored $[Ca^{2+}]_c$ and $[Ca^{2+}]_{ER}$ at various ER Ca^{2+} diffusion coefficients (D_{ER}) during the localised $[Ca^{2+}]_c$ oscillations. At a higher range (D_{ER}>20μm^2/s), a decrease in D_{ER} predominantly reduced the oscillation amplitudes, while at a lower range (D_{ER}<2 μm^2/s) both the amplitude and frequency of the oscillations were strongly affected by D_{ER}. We found that the effect of D_{ER} on the amplitude was due to the decrease in Ca^{2+} delivery to the releasing site during the rise of $[Ca^{2+}]_c$, while its effect on the frequency came from the delay in the recovery of $[Ca^{2+}]_{ER}$ in inter-spike periods. In the model with the high range of D_{ER}, a rise in $[InsP_3]$ predominantly increased the oscillation frequency, whereas in the model with the low range of D_{ER} the rise in $[InsP_3]$ caused an increase in the frequency as well as a decrease in the amplitude. The dose-response relationship in the model with the high D_{ER} is similar to the relationship in $InsP_3$-evoked Ca^{2+} oscillations experimentally shown in the acinar cells, suggesting that the ER Ca^{2+} diffusion coefficient in acinar cells falls into the high range of D_{ER} in the model. We conclude that intra ER Ca^{2+} diffusion has important effects on both the amplitude and the frequency of localised $[Ca^{2+}]_c$ oscillations and the effects are bifurcated by the Ca^{2+} diffusion coefficient in the ER.

Keywords: Pancreatic acinar cells, endoplasmic reticulum, computational model, Ca^{2+} oscillation, Ca^{2+} diffusion

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INDEX

BY AUTHOR

BY KEYWORD