8th Meeting of the European Calcium Society

Hinxton Hall, Cambridge, 28 – 31 July 2004

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Wednesday 28th July

13:00-19:00	Arrival and registration	
18:45-19:00 **Introduction**	Stephen Moss	
19:00-20:00 **Plenary Lecture** **Chair: Martin Bootman**	Donald Gill (Baltimore, USA)	Calcium entry mediated by SOCs and TRP channels: variations and enigma

20:00 - LATE - WELCOME PARTY

Thursday 29th July

SESSION 1: Mitochondria

Chair: Rosario Donato

8:30-9:15 **Keynote Lecture**	Rosario Rizzuto (Ferrara, It)	Mitochondrial Ca^{2+} signalling in cell life and death
9:15-9:45	Pontus Aspenstrom (Uppsala, S)	Rho GTPases in mitochondrial regulation
9:45-10:15	Mike Duchen (UK)	Calcium, mitochondria and oxidative stress in models of neurodegeneration

10:15-10:45 - BREAK

SESSION 2: Cell Division

Chair: Daniela Pietrobon

10:45-11:15	Michael Whitaker (Newcastle, UK)	Calcium signals in sperm, eggs and embryos
11:15-11:45	Howard Baylis (Cambridge, UK)	IP_3 signalling in the C. elegans embryo
11:45-12:15	John Carroll (London, UK)	Ca^{2+} signalling in meiosis and mitosis

12:15-15:30 - LUNCH AND POSTER SESSION 1

14:15-15:15	**SPONSORS**	**Technical Workshop**

SESSION 3: Ca^{2+}-binding proteins

Chair: Rachel Wong

15:30-16:00	Bob Burgoyne (Liverpool, UK)	Neuronal calcium sensor proteins and the regulation of neurotransmission
16:00-16:30	Walter Chazin (Nashville, USA)	Structural mechanisms and target selectivity in EF-hand proteins
16:30-17:00	Reg Morgan (Oviedo, Es)	Evolutionary perspective on calcium binding protein families

ORAL POSTER PRESENTATIONS

Chair: Jim Putney

17:00-17:15	Jan-Marek Weislogel (Heidelberg, Germany)	Calcium imaging using recombinant calcium probes in primary hippocampal neurons
17:15-17:30	Alexis Menteyne (Paris, Fr)	NAADP signalling in mice pancreatic acinar cells: characterization of the calcium stores involved
17:30-17:45	Gabriele Ackermann (Zurich, Switzerland)	S100A1-deficient mice display characteristics of hypertrophy and enhanced myocardial performance
17:45-18:00	David J Weber (Baltimore, USA)	The calcium-dependent interaction of S100B with p53 inhibits the function of the tumor suppressor

FREE EVENING

Friday 30th July

SESSION 4: Calcium and Disease I

Chair: Volker Gerke

8:30-9:15 **Keynote Lecture**	Harvey Pollard (Bethesda, USA)	Annexin-dependent signaling pathways
9:15-9:45	Wei-Jen Tang (Chicago, USA)	Structural basis for the regulation of anthrax edema factor by calcium-calmodulin
9:45-10:15	Daniela Pietrobon (Padova, It)	Mutant calcium channels and migraine

10:15-10:45 – BREAK

SESSION 5: Calcium Physiology

Chair: Claus Heizmann

10:45-11:15	Kathy Hajjar (New York, USA)	In vivo functions of annexin 2
11:15-11:45	Frank Wuytack (Leuven, B)	The Ca^{2+}/Mn^{2+} pumps in the Golgi apparatus
11:45-12:15	Mitsuhiko Ikura (Toronto, Can)	Structural basis for IP_3-mediated Ca^{2+} release from ER

12:15-15:30 - LUNCH AND POSTER SESSION 2

14:30-15:30 - ECS GENERAL ASSEMBLY

SESSION 6: Cell Signaling

Chair: Joachim Krebs

15:30-16:00	Jim Putney (Raleigh, USA)	Novel mechanisms of calcium entry regulation
16:00-16:30	Alexei Tepikin (Liverpool, UK)	How to make local calcium signals: a lesson from polarized epithelial cells
16:30-17:00	Vincenzo Sorrentino (Siena, It)	A molecular approach to the organization of the calcium releasing machinery in the sarcoplasmic reticulum

ORAL POSTER PRESENTATIONS

Chair: Jacques Haiech

17:00-17:15	Stéphanie Thebault (Villeneuve d'Ascq, Fr)	Differential role of TRP channels in Ca^{2+} entry and proliferation of primary human prostate cancer epithelial cells
17:15-17:30	Peter Lockyer (Cambridge, UK)	CAPRI is a novel sensor of Ca^{2+} entry revealing an analogue mode of Ca^{2+}-dependent ras deactivation
17:30-17:45	Thomas Vogl (Muenster, Germany)	Altered LPS signaling in $S100A9^{-/-}$ mice
17:45-18:00	Sacha Jensen (Oxford, UK)	Calcium binding to fibrillin-1 TB-cbEGF domain pairs: new insights into microfibril organisation

CONFERENCE BANQUET

Saturday 31st July

SESSION 7: Development and Neurobiology

Chair: Roland Pochet

9:00-9:45 **Keynote Lecture**	Rachel Wong (St.Louis, USA)	Calcium-dependent regulation of dendritic development in the retina
9:45-10:15	Nicholas Spitzer (San Diego, USA)	The frequency of calcium spikes specifies neurotransmitter expression in the spinal cord
10:15-10:45	Peter Mobbs (London, UK)	Calcium waves in the developing chick retina and retinal pigment epithelium

10:45-11:15 – BREAK

ORAL POSTER PRESENTATIONS

Chair: Alexei Tepikin

11:15-11:30	Catherine Leclerc / Marc Moreau (Toulouse, Fr)	A calcium-dependent pathway is involved in the formation of the embryonic kidney in Xenopus
11:30-11:45	Rod O'Connor (Cambridge, UK)	Cytosolic Ca^{2+} activates mitochondrial Ca^{2+} uptake
11:45-12:00	John Dedman (Ohio, USA)	Targeted inhibition of Ca^{2+}/calmodulin-dependent kinase II in cardiac myocytes of transgenic mice
12:00-12:15	Lorna Bailey (London, UK)	Roles for annexin 1 and annexin 2 in post-endocytic sorting of EGFR

12:15-13:45 - LUNCH

SESSION 8: Calcium and Disease II

Chair: Michael Berridge

13:45-14:15	Marie-France Bader (Strasbourg, Fr)	A role for lipids in the late stages of calcium-regulated exocytosis
14:15-14:45	Leon Lagnado (Cambridge, UK)	Calcium microdomains at the active zone
14:45-15:15	Xander Wehrens (New York, USA)	Ryanodine receptor dysfunction in cardiac disease

15:15 – 15:25 CLOSING REMARKS

15:25 MEETING ENDS

ABSTRACTS

RHO GTPASES IN MITOCHONDRIAL REGULATION

Pontus Aspenström

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The members of the Ras superfamily of small GTPases regulate a diverse panel of biological processes. During recent years, important links between Ras signalling and Ca^{2+} signalling have emerged. Ca^{2+}-dependent processes have been shown to influence Ras signalling. In addition, some newly identified members of the Ras superfamily were shown to have specific roles in Ca^{2+}-regulated pathways.

The Ras-like Rho GTPases have been shown to be key regulators of cell morphogenesis and cell migration. During a search for cDNAs encoding additional members of Rho GTPase family, a previously unidentified group of Rho GTPases with atypical features was identified. Studies employing immunofluorescence microscopy, revealed that members of this group were localised to mitochondria. For this reason, these proteins were named Miro (for mitochondrial Rho). Genes encoding Miro-like proteins were found in several eukaryotic organisms from *Saccharomyces cerevisae*, *Caenorhabditis elegans*, and *Drosophila melanogaster* to mammals, indicating that these genes evolved early during evolution. Miro GTPases have tandem GTP-binding domains separated by a linker region containing putative Ca^{2+}-binding EF hand motives. Interestingly, overexpression of constitutively active and dominant negative mutants of the human Miro-1 and Miro-2 in cultured cells induced a collapse of the mitochondrial network and resulted in an increased apoptotic rate of the cells. At present, the reason for these responses is not clear. However, some ideas about the mechanisms underlying the potential role of Miro in mitochondrial homeostasis and apoptosis will be discussed.

References:
Fransson, Å., Ruusala, A. and Aspenström, P. (2003). Atypical Rho GTPases have roles in mitochondrial homeostasis and apoptosis. *J Biol Chem* 278, 6495-6502.

Keywords : Rho, mitochondria, EF hands, apoptosis

CALCIUM SIGNALS IN SPERM, EGGS AND EMBRYOS

Michael Whitaker

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If a new individual is to appear as a consequence of sexual reproduction then sperm must find the egg, the egg must be activated at fertilization and the early embryonic cell cycles must be carefully regulated to allow differentiation to begin. Calcium signals are found to be involved with each of these regulatory events in the very early life of the individual.

Calcium imaging of single sea urchin sperm reveals calcium oscillations in the sperm tail which intensify in response to the putative chemotactic peptide speract[1].

A large wave of calcium increase crosses the cytoplasm at fertilization; it stimulates the egg to begin to divide[2]. I discuss how two calcium-releasing second messengers ($InsP_3$ and cyclicADPr) in sea urchin eggs combine to produce the calcium wave and the longer-lasting transient that it generates[3]. I also discuss the strengths and weaknesses of using the PLCδ plekstrin homology domain to measure $InsP_3$.

I describe the calcium signals that accompany mitosis in sea urchin and *Drosophila* embryos and show that interfering with $InsP_3$ signalling leads to cell cycle arrest.

Reference:
1. C.D. Wood, Alberto Darszon and Michael Whitaker (2003) Speract induces calcium oscillations in the sperm tail. J. Cell Biol. 161, 89-101.
2. Whitaker, M.J. (2003) Calcium signalling in eggs and embryos. Physiological Reviews *(in press)*
3. Calum Leckie, Ruth Empson, Andrea Becchetti, Justyn Thomas, Antony Galione and Michael Whitaker (2003) The NO pathway acts late during the fertilization response in sea urchin eggs. J Biol. Chem. 278, 12247-12254.

Keywords: calcium, fertilization, sperm, chemotaxis, cell cycle, $InsP_3$, cyclicADPr

NEURONAL CALCIUM SENSOR PROTEINS AND THE REGULATION OF NEUROTRANSMISSION

Robert D Burgoyne

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Many aspects of neuronal activity are regulated by calcium signals. The transduction of temporally and spatially distinct calcium signals requires the action of calcium sensor proteins including various EF-hand containing calcium-binding proteins. The Neuronal Calcium Sensor (NCS) family proteins have begun to emerge as key players in the regulation of neuronal function (Burgoyne RD et al., Trends Neurosci 2004; 27:203-209). Many of these proteins are expressed predominantly or only in neurons sometimes with cell-specific patterns of expression. The NCS protein family is encoded by 14 genes in the human genome that possess four EF hand domains of which 2 or 3 bind calcium. The NCS proteins are high-affinity calcium-binding proteins that act as calcium sensors rather than calcium buffers as they undergo conformational changes on calcium-binding and regulate target proteins. Many NCS proteins are N-terminally myristoylated and their ability to associate with membranes either constitutively or in response to calcium elevation (the calcium/myristoyl switch) allows the NCS proteins to discriminate between different spatial and temporal patterns of calcium signals (O'Callaghan DW et al., J Biol Chem 2002; 277:14227-14237; O'Callaghan DW et al., J Cell Biol 2003; 163:715-721; O'Callaghan DW et al., J Cell Sci 2003; 116:4833-4845). Recent work has established several physiological roles of these proteins relating to the regulation of neurotransmission in some cases by direct effects on channel function or their traffic to the cell surface. Some of the characteristics and functional diversity of the NCS proteins will be illustrated from our studies on NCS-1, hippocalcin and KChIP1.

Key words: NCS-1, frequenin, hippocalcin, KChIP, channels

STRUCTURAL MECHANISMS AND TARGET SELECTIVITY IN EF-HAND PROTEINS

Shibani Bhattacharya, Haitao Hu, Melanie Nelson, Christopher Bunick, Walter J. Chazin

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EF-hand calcium binding proteins have remarkable sequence homology and structural similarity, yet their response to binding of calcium is diverse and they function in a wide range of biological processes. Knowledge of how nature fine tunes EF-hand protein sequence to optimize specific biochemical properties has been significantly advanced over the past 10 years with the determination of many 3D structures at atomic resolution. These data lay the foundation for addressing the challenge of understanding how functional selectivity is generated from a generic ionic signal.

Our efforts are focused on the structural mechanisms that provide the selectivity of different EF-hand proteins for specific cellular targets. There are three factors that contribute to target selectivity: molecular architecture, response to the binding of Ca^{2+} ions, and the characteristics of target binding surfaces. S100 and calmodulin family proteins will be used to demonstrate these critical concepts (1,2). A detailed comparison of calmodulin and centrin will be presented to show how these factors can be combined to generate selectivity for a specific protein target, even though these two proteins that have remarkably similar structures (3).

References:
1. S. Bhattacharya, E. Large, C.W. Heizmann, B. Hemmings and W.J. Chazin. "Structural mechanism of calcium dependent regulation of the NDR Kinase by S100B." Biochemistry 42, 14416-14426 (2003).
2. C.G. Bunick, M.R. Nelson, S. Mangahas, M.J. Hunter, J.H. Sheehan, L.S. Mizoue, G.J. Bunick and W.J. Chazin. "Designing sequence to control function in an EF-hand Protein." J. Amer. Chem. Soc. 126, 5990-5998 (2004).
3. H. Hu and W.J. Chazin. "Unique Features in the C-terminal Domain Provide Caltractin with Target Specificity." J. Mol. Biol. 330, 473-484 (2003).

Keywords: signal transduction, calmodulin, centrin, S100, homology

EVOLUTIONARY PERSPECTIVE ON CALCIUM BINDING PROTEIN FAMILIES

Reginald O. Morgan

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The molecular evolution of calcium-binding domains (CBDs) can be traced throughout all branches of life to define phylogenetic relationships for classification, to elaborate models of structural variation, and to discern functional elements and mechanisms. Phylogenetic analysis of the principal CBDs (e.g. C2, EF-hand, annexin type 2) revealed clade formations and divergent branches indicative of significant evolutionary events in the creation, demise and remodeling of CBD within selected protein families. Structural comparisons were facilitated by the elaboration of hidden Markov models for individual protein subfamilies, to conceptualize distinct molecular profiles such as the prokaryotic excalibur domain, and to search, align and classify novel homologs. The context-sensitive nature of bacterial CBDs and the modular architecture characteristic of higher eukaryotes have contributed to evolutionary, structural and functional diversity of the calcium-binding property in different protein families. Parameters of site-specific conservation and unusual evolutionary rate changes in CBD were computed to identify key functional elements and saltatory adaptations. This evolutionary information was incorporated into 3D crystallographic models for visualization and docking interaction trials.

Punctual changes in CBD evolution have accompanied major speciation events and geoclimatic upheavals, highlighting the role of genome remodeling in the macroevolution of CBDs. This is exemplified by the loss of calcium-binding proteins from yeasts, the Cambrian genome duplications prescribing the vertebrate endoskeleton, phytoplankton exoskeletons that whitened the cliffs of Dover, and the gene cluster expansions of S100 in human 1q21 and the secretory calcium-binding phosphoproteins at 4q13. Nature has provided us with a cornucopia of site-directed mutagenesis and natural knockouts of CBD to observe, interpret and infer.

Calcium's unique chemical properties make it ideally suited for controlling protein structure and conformation, often in a synergistic manner with other charged structures such as phospholipids. Thus, the transport targeting role of calcium may be just one aspect of protein function, and subsequent convergence of calcium-dependent membrane binding proteins could lead to other, more specific interactions within protein complexes collectively engaged in adhesion, trafficking, remodeling, signaling or transcription functions. The ablation of calcium binding sites in certain annexin proteins, the identification of other putative interaction sites by evolutionary analyses, and the frequent union of annexins with S100 and C2-containing proteins suggest a coordinated, but still elusive, physiological role.

References:
1. Fernandez MP, Morgan RO (2003). Structure, function and evolution of the annexin gene superfamily. In "The Annexins: Biological Importance and Annexin-related Pathologies", pp.21-37, J. Bandorowicz-Pikula,ed., Landes.
3. Moss SE, Morgan RO (2004). The annexins. Genome Biol 5: 219.1-219.8.

Keywords: calcium-binding domains, hidden Markov models, molecular evolution, phylogenetic analysis.

NOVEL MECHANISMS OF CALCIUM ENTRY REGULATION

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In non-excitable cells, the release of Ca^{2+} from intracellular stores by inositol 1,4,5-trisphosphate (IP_3) and other intracellular Ca^{2+} mobilizing signals is often coupled to the entry of Ca^{2+} across the plasma membrane, a process called capacitative calcium entry or store-operated calcium entry. Recently, evidence has surfaced for other modes of regulated entry in non-excitable cells. In neither case is the molecular nature of the channels known. We have investigated the behavior and regulation of TRPC channels, specifically with regard to their gating by store-operated and non-store-operated mechanisms. Our findings indicate that two of the TRPCs, specifically TRPC3 and TRPC7 can function either as store-operated or as second messenger (likely diacylglycerol) operated channels, depending on expression conditions. In addition, we have found that cellular protein kinases regulate the activity of these channels. Protein kinase C provides a negative feedback by diacylglycerol by phosphorylating TRPC3 and inhibiting its activity. On the other hand, Src kinase is a positive regulator of TRPC3 and is required for activation of the channel whether by receptor agonists or more directly by synthetic diacylglycerol.

Keywords: Calcium entry; TRPC channels; Store-operated calcium entry; Protein kinase C; Src kinase

MUTANT CALCIUM CHANNELS AND MIGRAINE

Daniela Pietrobon

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Familial hemiplegic migraine type 1 (FHM-1) is a subtype of migraine with aura that is caused by missense mutations in the *CACNA1A* gene that encodes the alpha$_1$ subunit of neuronal $Ca_v2.1$ Ca^{2+} channels. Functional studies into FHM may improve the insight into the pathophysiology of migraine, a debilitating illness that afflicts 10-15% of the population. The functional consequences of FHM-1 mutations have been first investigated by expressing recombinant human $Ca_v2.1$ channel subunits in heterologous systems and in cerebellar granule cells from *cacna1a*$^{-/-}$ mice. These studies revealed two apparently contradictory functional effects common to all FHM-1 mutations analyzed: gain-of-function at the single channel level and loss-of-function at the whole-cell level due to reduced density of functional channels in the membrane (Tottene et al., 2002, Proc. Natl. Acad. Sci. 99, 13284-89). To study the consequences of FHM-1 mutations on neuronal $Ca_v2.1$ channels at their endogenous level of expression and the consequences of mutant $Ca_v2.1$ channels on cortical spreading depression (CSD: the likely mechanism for the migraine aura), a knock-in (KI) mouse model carrying the FHM-1 R192Q mutation was generated. Patch-clamp recordings on cerebellar neurons in primary culture revealed an increased $Ca_v2.1$ Ca^{2+} current density in the KI mouse, as a consequence of mutant channels that open more readily and at lower voltages than wild-type (wt) channels. The density of functional $Ca_v2.1$ channels was similar in neurons of KI and wt mice. Gain-of-function of neuronal $Ca_v2.1$ channels resulted in a lowered threshold for induction and an increased velocity of propagation of CSD in the intact animal. Our data show an important role of $Ca_v2.1$ channels in the initiation and spread of CSD, and point to cortical hyperexcitability as the basis for increased susceptibility to CSD in migraine. The R192Q FHM-1 mouse is a promising animal model to study migraine mechanisms and treatments.

THE Ca^{2+}/Mn^{2+} PUMPS IN THE GOLGI APPARATUS

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The SPCAs (secretory pathway Ca^{2+}-ATPases; gene *ATP2C1*; 3q21-q24) represent the latest discovered subfamily of P-type Ca^{2+}-transport ATPases. SPCA1 appears to be a house-keeping enzyme in the Golgi apparatus and possibly also in some of the more distal compartments of the secretory pathway. It pumps, in a thapsigargin-insensitive manner, one Ca^{2+} ion per cycle into the Golgi with an affinity comparable to SERCA2. SPCA1 can also accumulate Mn^{2+} with a similar affinity. Four different splice variants (SPCA1a-d) are known differing only in their C-termini. SPCA1c with an incomplete C-terminal transmembrane segment is not a functional Ca^{2+} pump.

Loss of one functional *ATP2C1* allele causes Hailey-Hailey disease, a skin disorder in adult age with formations of blisters and erosions in the flexural areas.
Expression of some of the Hailey-Hailey mutants (L341P, C344Y, T570I, G789R) in COS-1 cells suggests increased protein instability. G309C and D742Y are defective in the Ca^{2+}- or Mn^{2+}-dependent formation of the phosphoprotein intermediate. I580V phosphorylates normally, but presents impaired dephosphorylation.

From the *ATP2C2* homologue (16q24.1) the sequence of yet another putative Ca^{2+} pump (SPCA2) can be deduced. Like SPCA1, SPCA2 is predominantly found in the Golgi, but unlike SPCA1 it has a more restricted tissue distribution (secretory epithelia, neuronal cells). Whether SPCA2 functions as a Ca^{2+} and/or Mn^{2+} pump is not clear.

STRUCTURAL BASIS FOR IP_3-MEDIATED Ca^{2+} RELEASE FROM ENDOPLASMIC RETICULUM

I. Bosanac[1], J. Chan[1], T. K. Mal[1], F. Yoshikawa[2], T. Michikawa[2], M. Mikoshiba[2], M. Ikura[1]

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[2]Institute of Medical Science, University of Tokyo, Tokyo and RIKEN Brain Science Institute, Wako, Japan

A central mechanism for calcium (Ca^{2+}) regulated cellular processes involves release of Ca^{2+} from the intracellular endoplasmic reticulum (ER) Ca^{2+}-store into the cytoplasm. In a variety of cells, this Ca^{2+} signalling process is mediated by the ER membrane-associated Ca^{2+} release channel, inositol 1,4,5-trisphosphate receptor (IP_3R). As a signal transducer between the two ubiquitous second messengers D-*myo*-inositol 1,4,5-trisphosphate (IP_3) and Ca^{2+}, IP_3R plays a crucial role in the control of cellular and physiological processes as diverse as cell division, cell proliferation, apoptosis, fertilization, development, behavior, memory and learning. We have recently determined a crystal structure of the IP_3 binding core of the mouse type I IP_3 receptor at 2.2 Å (*Nature* 420, 696-670, 2002). The asymmetric, boomerang-like structure consists of an N-terminal β-trefoil domain and a C-terminal α-helical domain containing an armadillo repeat fold. The cleft formed by the two domains exposes a cluster of arginine and lysine residues that coordinate the three phosphoryl groups of IP_3. In light of this crystal structure as well as EM structures of the tetrameric receptor under different conditions published recently by various groups, the talk will discuss possible mechanisms underlying how the second messenger IP_3 initiates the gating function of the Ca^{2+} release channel in collaboration with another key agonist Ca^{2+}.

Keywords: calcium signaling, IP3, IP3 receptor, X-ray, crystal structure

A MOLECULAR APPROACH TO THE ORGANIZATION OF THE CALCIUM RELEASING MACHINERY IN THE SARCOPLASMIC RETICULUM

Vincenzo Sorrentino

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The endo-sarcoplasmic reticulum of striated muscle cells consists of distinct functional domains that are extremely well organised both in terms of functional specialisation and of spatial organisation. Indeed, the sarcoplasmic reticulum is able to maintain a highly organised relationship with the myofibrils, resulting in the formation of a sleeve-like structure of intracellular membranes organised around each myofibril that is critical for muscle contraction. Furthermore how specific proteins, like calcium release channels and calcium pumps, are redistributed within the sarcoplasmic reticulum in order to organise functionally distinct domains and how this structural organisation of the sarcoplasmic reticulum aligns with respect to specific regions of the sarcomere is not understood. Increasing evidence indicates that cytoskeletal proteins, including ankyrins and spectrins, may participate in the organisation of intracellular organelle membranes and, possibly, in the positioning of specific proteins on the surface of these organelles. In this context, we reported that ank1.5, a striated muscle-specific isoform of the Ankyrin 1 gene located on the sarcoplasmic reticulum membrane, and Obscurin, a giant protein associated with the myofilaments, may, through their direct interaction, play a role in the alignment of the sarcoplasmic reticulum with respect to the sarcomere. Recent developments aiming to dissect the specific interactions that may have a role in the organization of the sarcoplasmic reticulum will be discussed.

References:
Bagnato P., Barone V., Giacomello E., Rossi D. and Sorrentino V. Binding of an Ankyrin-1 isoform to the C-terminus of Obscurin identifies a molecular link between the sarcoplasmic reticulum and myofibrils in striated muscles. 2003. J. Cell. Biology 160,245-253.

Keywords: sarcoplasmic reticulum, myofibrils, Calcium release, ryanodine receptors, $InsP_3$ receptors, endoplasmic reticulum

CALCIUM DEPENDENT REGULATION OF DENDRITIC DEVELOPMENT IN THE RETINA

R.O.L. Wong

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Neural circuits in the vertebrate retina are established during development by the growth and restructuring of presynaptic (axons) and postsynaptic (dendrites) structures.
In order to better understand how neural connections are formed, maintained or eliminated as retinal circuits are assembled, we are investigating the cellular mechanisms that organize axonal and dendritic structure during development. I will focus primarily on our time-lapse live imaging studies of dendritic development of retinal ganglion cells, the output neurons of the retina. The dendritic arbors of functionally distinct retinal ganglion cells stratify in separate layers or laminae where they are contacted by specific populations of presynaptic cells. The dendrites of immature retinal ganglion cells undergo growth and remodeling before becoming stratified; if they fail to do so, they receive abnormal connections at maturity. I will discuss the role of calcium-signalling pathways that regulate dendritic outgrowth and remodelling of the ganglion cells. In particular, I will contrast the roles of global changes in intracellular calcium levels and local calcium elevations that control separate aspects of dendritic development and plasticity in ganglion cells.

Reference:
R.O.L. Wong and A. Ghosh (2002) Nat. Neurosci. Rev. 3:803-812.

Key words: synaptogenesis, calcium-induced calcium release

ACTIVITY-DEPENDENT HOMEOSTATIC SPECIFICATION OF TRANSMITTER EXPRESSION IN EMBRYONIC NEURONS

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Neurotransmitters are essential for interneuronal signaling, and specification of appropriate transmitters in differentiating neurons has been related to intrinsic neuronal identity and to extrinsic signaling proteins. Here we show that altering the distinct patterns of Ca^{2+} spike activity spontaneously generated by different classes of embryonic spinal neurons *in vivo* changes the transmitter neurons express without affecting expression of markers of cell identity. Regulation appears to be homeostatic: suppression of activity leads to an increased number of neurons expressing excitatory transmitters and a decreased number of neurons expressing inhibitory transmitters; the reverse occurs when activity is enhanced. Imposition of specific spike frequencies *in vitro* does not affect labels of cell identity but again specifies expression of transmitters that are inappropriate for the markers they express, during an early critical period. The results identify a new role of patterned activity in development of the central nervous system.

Keywords : calcium spikes, critical period, neuronal differentiation, neurotransmitters, spinal cord

A ROLE FOR LIPIDS IN THE LATE STAGES OF CALCIUM-REGULATED EXOCYTOSIS

Marie-France Bader, Sylvette Chasserot-Golaz and Nicolas Vitale

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Calcium-regulated exocytosis of hormones and neurotransmitters is the topic of intense investigations and many proteins that catalyze targeting and fusion of secretory vesicles with the plasma membrane are now identified. However, the lipid composition of vesicles and their target membrane is also critical and lipid modifications may be required in several stages of the exocytotic pathway. Phospholipase D (PLD) generates phosphatidic acid (PA), a multifunctional lipid that can activate selected enzymes, serve as protein attachment site or alter membrane curvature. In chromaffin and PC12 cells, PLD1 is present at the plasma membrane and is activated, upon secretagogue-evoked stimulation, by the secretory granule-associated ADP-ribosylation factor 6 (ARF6) (1). Expression of a catalytically inactive PLD1 mutant in PC12 cells strongly inhibits secretion, supporting a major role for PLD1 in the exocytotic pathway. We microinjected the inactive PLD1 mutant into chromaffin cells and monitored catecholamine release by amperometry. Inactive PLD1 not only reduced the number of release events (spikes) but it also affected the characteristics of the remaining spikes, consistent with a possible post-docking function for PLD1 (2). We propose that the PLD1-induced local elevation of PA at exocytotic sites, by promoting membrane bending, facilitates hemi-fusion and subsequent formation and/or expansion of the exocytotic fusion pore.

Using deletion and mutagenesis analysis, we found that the PX domain and $PI4,5P_2$-binding site are critical for the association of PLD1 to the plasma membrane in resting cells. However, in secretagogue-activated cells, PLD1 lacking palmitoylation falls into the cytoplasm and fails to activate exocytosis, suggesting that a translocation step into lipid rafts is required for PLD1 to function in exocytosis (3). Biochemical and immunological studies confirm the calcium-dependent formation of cholesterol and ganglioside-enriched lipid rafts in the plasma membrane of secretagogue-stimulated chromaffin cells. These rafts co-localize with sites of active exocytosis and seem to be stabilized by annexin 2, a cytosolic calcium and lipid-binding protein known to translocate to the plasma membrane upon cell stimulation. Thus, exocytotic sites may be determined by the local formation of lipid micro-domains required for the structural and spatial organization of the many components involved in exocytosis. Among them, PLD1 seems to play a decisive role in the late stages of exocytosis, most likely by adding specific lipid modifications required for the membrane fusion machinery to function.

References:

1. Vitale N., Chasserot S., Bailly Y., Morinaga N., Frohman M.A. & Bader M.F. (2002) J. Cell Biol. 159: 79-89.
2. Vitale N., Caumont A.S., Chasserot-Golaz S., Du G., Wu S., Sciorra V.A., Morris A.J., Frohman M.A. & Bader M.F. (2001) EMBO J. 20: 2424-2434.
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Keywords: exocytosis - lipid micro-domains - phospholipase D – annexin 2 – ARF6 – chromaffin cells

RYANODINE RECEPTOR DYSFUNCTION IN CARDIAC DISEASES

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Altered cardiac ryanodine receptor (RyR2) function has an important role in heart failure and genetic forms of arrhythmias. RyR2 constitutes the major intracellular Ca^{2+} release channel in the cardiac sarcoplasmic reticulum (SR). The petidyl-prolyl isomerase calstabin2 (FKBP12.6) is an important regulatory component of the RyR2 macromolecular signaling complex. Calstabin2 binding to RyR2 is regulated by PKA phosphorylation of Serine 2809 in RyR2. PKA phosphorylation of RyR2 decreases the binding affinity for calstabin2, increases RyR2 open probability and sensitivity to Ca^{2+}-dependent activation.

Evidence has accumulated over the past five years that abnormal calcium release via ryanodine receptors contributes to impaired calcium cycling. Chronic hyperactivity of the sympathetic nervous system during heart failure results in protein kinase A hyperphosphorylation of the cardiac ryanodine receptor, which leads to dissociation of the stabilizing subunit calstabin2 from the channel macromolecular complex. Calstabin2-depleted channels can leak calcium from the sarcoplasmic reticulum (SR) during diastole (when the channels are supposed to be tightly closed). This diastolic SR calcium leak can deplete SR calcium and contribute to contractile dysfunction as well as trigger fatal cardiac arrhythmias.

Missense mutations in the human RyR2 gene have been linked to genetic forms of exercise-induced cardiac arrhythmias and sudden cardiac death. These RyR2 mutations also cause decreased calstabin2 binding affinity and leaky RyR2 channels following PKA phosphoryation. These new observations implicate dysregulation of calstabin2 binding as a central mechanism for abnormal calcium cycling in heart failure and triggered arrhythmias. Promising new therapeutic agents are currently being developed to inhibit the diastolic SR calcium leak in heart failure or patients with arrhythmias, by enhancing the affinity of calstabin2 for the ryanodine receptor.

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Keywords : Arrhythmias, calcium, calstabin2, heart failure, ryanodine receptor.

Ca^{2+}/CALMODULIN REGULATION OF αCaMKII TRANSLOCATION IN HIPPOCAMPAL NEURONES

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The neuronal Ca^{2+}/calmodulin-dependent protein kinase II (αCaMKII) responds to Ca^{2+} stimulation by translocation into particulate subcellular fractions both as a result of physiological stimulation[1,2] and hypoxia[3]. The mechanism of αCaMKII translocation was investigated in hippocampal neurones using EGFP-tagged calmodulin and αCaMKII and confocal microscopy. Intracellular [Ca^{2+}] elevation was evoked by extracellular Ca^{2+}/ionomycin. Fluo-4 fluorescence rose 24 (± 9)-fold with a $t_{1/2}$ of 10 (± 3) s in the cytosol and dendrites and remained elevated throughout the experiment. Prior to stimulation, both endogenous CaMKII detected by immuno-cytochemistry and EGFP-αCaMKII were homogenously distributed throughout the cytoplasm. Following stimulation, both the endogenous and exogenous αCaMKII translocated into punctate structures in both the cell soma and in the dendritic processes with a lag of 138 (± 12) s. The development of the punctate pattern was complete within 460 (± 34) s and persisted for > 30 min. EGFP-CaM, upon Ca^{2+} stimulation displayed two major responses: one fraction of EGFP-calmodulin entered the nucleus and the other co-localised with αCaMKII into punctate structures.

The dynamics of calmodulin and αCaMKII translocation were investigated using a cell permeant calmodulin inhibitor, mTrp peptide. mTrp inhibited translocation at a concentration of 13 μM, however, at 6.5 μM, mTrp, initially allowing αCaMKII translocation, induced the dissociation of the punctate structures. This dynamic stage of translocation was followed by a persistent stage: 30 min after translocation neither EGTA, nor the mTrp peptide was able to disrupt the punctuate structures. Immunocytochemical detection of the NR2B subunit of the NMDA receptor[4] showed that the NR2B subunit was present at the plasmamembrane of the hippocampal neurones, it however did not co-localise with the punctuate Ca^{2+}/calmodulin/αCaMKII structures suggesting that these were formed by self-association of Ca^{2+}/calmodulin/αCaMKII complexes.

Our data show biphasic regulation of αCaMKII translocation by Ca^{2+}/calmodulin and demonstrate that dynamic association of Ca^{2+}/calmodulin and αCaMKII is followed by an irreversible stage. Ca^{2+}/calmodulin.αCaMKII self-association may be relevant in pathological conditions involving persistent elevation of intracellular [Ca^{2+}].

This work is supported by the MRC and the Wellcome Trust.

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Keywords : αCaMKII, calmodulin, hippocampal neurone

CALCIUM IMAGING USING RECOMBINANT CALCIUM PROBES IN PRIMARY HIPPOCAMPAL NEURONS

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The generation of calcium signals following synaptic activity is a fundamental property of neurons that controls many neuronal processes including the regulation of gene expression. For synapse-to-nucleus communication, neurons exploit the spatial and temporal diversity of calcium transients associated with electrical activation. Dendritic calcium signals, activating the ERK-MAP kinase signaling cascade, stimulate gene expression mediated by the serum response element. Increases in the nuclear calcium concentration are critical for the activation of gene expression mediated by the transcription factor CRE-binding protein (CREB) and the coactivator CREB-binding protein (CBP) (1). The CREB/CBP complex has been implicated in learning and memory and neuronal survival, indicating that nuclear calcium may be a key regulator of transcription–dependent neuronal adaptation. To develop calcium indicators that allow us to monitor calcium signals in distinct subcellular compartments, particularly in the cell nucleus, we have been using the recombinant calcium probes Inverse Pericam (2) and G-CaMP (3). Either calcium probe allowed the detection of global calcium transients evoked by KCl-induced membrane depolarization in primary rat hippocampal neurons. G-CaMP was also suitable for monitoring slow calcium oscillations triggered by recurrent burst activity in a hippocampal network. To target G-CaMP to the nucleus a nuclear localization signal was fused to the amino terminus of G-CaMP. The results obtained with the NLS-G-CaMP will be discussed.

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Keywords: calcium signaling, recombinat calcium probes

THE SPECIFIC INTRACELLULAR LOCALISATIONS OF NCS PROTEINS MAY BE MEDIATED BY THEIR DIRECT INTERACTION WITH MEMBRANE PHOSPHOINOSITIDES

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The Neuronal Calcium Sensor proteins are important signalling proteins that transduce intracellular calcium signals and modulate membrane lipid signalling[1]. The intracellular localisations of members of the NCS family have been examined in hippocampal neurones and HeLa cells. It was found that both NCS-1 and hippocalcin are distributed to the trans-Golgi network (TGN) and regions of the plasma membrane whilst KChIP-1 is localised to post-ER trafficking vesicles[2]. These localisations are mediated by the N-terminal myristoyl groups of these proteins and the differential distribution is determined by residues in the myristoylation sequence. Green Fluorescent Protein (GFP) variants tagged with the myristoylation sequences of hippocalcin, NCS-1 or KChIP-1 localised to the same region as the full length protein. The differential localisation of these myristoylation sequences was due to the presence or absence of charged residues within these regions. A myristoylated peptide corresponding to the hippocalcin myristoylated sequence when added to permeabilised cells became co-localised with NCS-1. Lipid blot assays demonstrated that this myristoylation sequence of hippocalcin interacted specifically with lipids containing phosphoinositide head groups. No binding was seen with either non-myristoylated peptide or the KChIP-1 myristoylated peptide. These peptides also interacted specifically with liposomes containing phosphoinositides in pull down assays. Phosphatidylinositol-specific pleckstin homology domains have been used to probe the specificity of the lipid targeting of hippocalcin to particular phospholipids. The KChIP-1 myristoylation sequence did not interact directly with any lipids suggesting that its localisation is likely to be mediated by protein interactions. These data indicate that membrane phosphoinositides are important for hippocalcin and NCS-1 but not KChIP-1 localisation.

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Keywords : NCS-1, myristoylation, hippocalcin, phospholinositide

THE NEURONAL CALCIUM SENSOR VILIP-1: INVOLVEMENT IN HIPPOCAMPAL PATHOPHYSIOLOGY OF SCHIZOPHRENIA

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Post mortem studies in the schizophrenic hippocampus revealed a decreased expression of VILIP-1 in pyramidal neurons and an increased number of VILIP-1-positive interneurons (1, 3). Similar results have been observed in the hippocampus of ketamine-treated rats, a glutamatergic hypofunction model for schizophrenia (2). Interestingly, the expression of VILIP-1 is also regulated in a form of hippocampal synaptic plasticity, long-term potentiation, and depends on glutamatergic transmission (4). These findings raise the question of the regulation and the possible role of VILIP-1 in pathophysiological processes in the hippocampus probably leading to cognitive deficits. To address these questions we have examined the influence of glutamatergic neurotransmission on VILIP-1 expression by immunocytochemistry which allowed to distinguish cell specific expression in pyramidal neurons and interneurons. Glutamate leads to neurotoxicity in pyramidal neurons and interneurons which is inhibited by addition of the NMDA receptor antagonist MK-801. Glutamate also leads to an increase in the number of VILIP-1-positive interneurons. The use of DHPG, 4CPG and MPEP, specific mGluR group I agonist and antagonists, shows that mGluR1α regulates the glutamate-dependent increase in VILIP-1-expression in interneurons. Since mGluRs are known to regulate synaptic plasticity in the hippocampus in vivo the observed pathological changes in VILIP-1 expression in interneurons may be related to synaptic plasticity and may partially explain negative symptoms in schizophrenia including learning and memory deficits.

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Keywords : glutamatergic hypofunction; hippocampus; interneurons; mGluR; NCS protein; schizophrenia

SURAMIN INHIBITS THE INOSITOL 1,4,5-TRISPHOSPHATE RECEPTOR BY INTERACTING WITH THE N-TERMINAL CALMODULIN-BINDING SITE

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Calmodulin (CaM) is a ubiquitous Ca^{2+}-sensor protein that plays an important role in regulating a large number of Ca^{2+} channels, including the inositol 1,4,5-trisphosphate receptor (IP_3R). CaM binds to the IP_3R at Ca^{2+}-dependent as well as at Ca^{2+}-independent interaction sites. CaM was found to inhibit IP_3R function only in the presence of Ca^{2+}, thereby modulating the Ca^{2+}-dependent bell-shaped activation curve of the IP_3R, while no function has yet been found for the Ca^{2+}-independent interaction (1,2). Suramin, a polysulphonated naphtylurea, displaced CaM both in the presence and absence of Ca^{2+}. Suramin competed with CaM for binding to different peptides representing the previously identified CaM-binding sites on IP_3R1. By interacting with the N-terminal Ca^{2+}-independent CaM-binding site, suramin mimicked the functional effect of CaM and induced an allosteric but competitive inhibition of IP_3 binding. As a consequence suramin also potently inhibited IP_3-induced Ca^{2+} release (IICR) from permeabilized cells predominantly expressing IP_3R1 (L15 fibroblasts) or IP_3R3 (Lvec fibroblasts). Furthermore, we have found that CaM_{1234}, a CaM mutated in its four EF hands, inhibited IICR in a Ca^{2+}-dependent way with the same potency as CaM. We conclude that CaM inhibits IICR via the N-terminal binding site. The inhibition requires Ca^{2+} but CaM itself is not the Ca^{2+} sensor for the inhibition of the IP_3R.

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Key words: IP_3R, calmodulin, suramin

INOSITOL 1,4,5-TRISPHOSPHATE ($INSP_3$) AND ITS RECEPTOR ARE REQUIRED FOR STORE OPERATED CALCIUM ENTRY

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Calcium depletion from intracellular stores results in calcium entry across the plasma membrane, a process commonly termed store operated calcium entry (SOCE). The calcium entry is used to replenish the intracellular calcium stores and regulate cellular processes such as gene transcription and secretion [1]. The mechanism by which the state of filling of the ER is transduced to the plasma membrane remains unclear. A direct conformational coupling between the $InsP_3R$ and plasma membrane influx channels, or a diffusible messenger are both hypothesised to be involved [2]. Previous studies have reported a role for both $InsP_3$ and the $InsP_3R$, however such studies are compromised by the poor pharmacology of the $InsP_3$ signalling cassette. In this study we used molecular techniques to manipulate $InsP_3$ and $InsP_3R$ levels. We found that increasing $InsP_3$ levels by overexpressing phospholipase C (PLC) or phosphatidylinositol (PI) 4-OH kinase resulted in a significant enhancement of SOCE. Conversely, reducing $InsP_3$ levels by decreasing PLC or PI4-OH kinase activities resulted in a significant lowering of SOCE. Use of an $InsP_3$ sequestering 'sponge' and $InsP_3$ metabolising 5'-phosphatase also significantly decreased SOCE. To investigate the role of the $InsP_3R$, the type 3 and type 1 isoforms were stably knocked down by RNA interference while the type 2 isoform was transiently knocked down. We found that ablation of either the type 1 or type 3 $InsP_3R$ resulted in a significant decrease in SOCE whereas reduction in the expression of the type 2 $InsP_3R$ had no effect. We propose that both $InsP_3$ and $InsP_3Rs$ are critical components in the mechanism of SOCE.

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Keywords: SOCE, $InsP_3$, $InsP_3R$, calcium

NAADP SIGNALLING IN MICE PANCREATIC ACINAR CELLS: CHARACTERIZATION OF THE CALCIUM STORES INVOLVED

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Intracellular Ca^{2+} levels play a key role in several cellular processes, including secretion. Aside from the well established Ca^{2+} releasing messengers IP3 and cADPr (3) , a new Ca^{2+} releasing messenger, NAADP has been recently discovered (1). The NAADP transduction pathway is poorly understood. For example, its receptor hasn't been cloned yet and the identity of the Ca^{2+} store mobilized is still under investigation. Lysosome-like organelles have been proposed to be NAADP sensitive in sea urchin eggs (2) and pancreatic acinar cells(4). To test the hypothesis that lysosomes are a Ca^{2+} store recruted by NAADP, Ca^{2+} or agonists, we have simultaneously recorded Ca^{2+} signals and lysosomes distribution using confocal microscopy. Lysosomes have been visualized by the dye Lysotracker red and specifically disrupted by application of Gly-Phe-β-naphtylamide (GPN), a specific substrate of the lysosomal enzyme Cathepsin C, that provokes osmotic swelling of these organelles.

Our data show that:
- Lysosomes labelled using the Lysotracker red were disrupted by GPN. The lysis of lysosomes provoked a Ca^{2+} release that did not affect the endoplasmic reticulum Ca^{2+} stores (ER). Store characterization experiments were repeated in Ca^{2+} free media and showed similar results, indicating that the cytosolic Ca^{2+} increase evoked by GPN treatment originated from an intracellular store.
- We have observed that CCK or ACh stimulation induced lysosomes mobilisation. Interestingly, lysosomes were also mobilized when a Ca^{2+} elevation was induced by ER depletion using the SERCA pump inhibitor thapsigargin (Tg).
- We have used GPN to test the involvement of lysosomes as a Ca^{2+} store in cholecystokinin (CCK), Acetylcholine (Ach), and NAADP signalling. Two concentrations of GPN were tested. A low dose of GPN (50 µM) evokes a small release of Ca^{2+} due to lysosomes lysis and selectively disrupt the Ca^{2+} response to 5 pM CCK, whereas Ach at 25 nM evokes a normal Ca^{2+} response as previously reported (4). However, addition of a higher dose of GPN (200 µM) induced an even more important Ca^{2+} release, and prevented the Ca^{2+} response to 25 nM Ach, suggesting that lysosomes are required also for Ach signalling.
In addition, we have investigated the effect of lysosomes lysis on the NAADP response. Our data show that 200 µM GPN abolishes NAADP Ca^{2+} response. In regard to our previous finding that NAADP is involved in CCK-evoked Ca^{2+} signals, these data suggest that ACh and CCK may recruit lysosomes through NAADP pathway, and ACh through ER Ca^{2+} mobilisation.

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Keywords : Acinar cell; NAADP; lysosome; Cholecystokinin; Acetylcholine.

THE TRPM2 AGONIST ADP-RIBOSE ACTS AS SECOND MESSENGER

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Introduction: Adenosine-diphosphoribose (ADPR) is involved in the regulation of Ca^{2+} influx into cells by gating the Ca^{2+} permeable ion channel TRPM2 [1-2]. Since no method is available to determine cellular APDR concentrations, it is unclear whether ADPR is a modulator or second messenger under physiological conditions. The aims of the present study were to establish an assay for the quantification of intracellular ADPR and to analyse the causal relationship between ADPR formation and Ca^{2+} entry.

Materials and methods: To determine cellular ADPR levels a protocol consisting of acidic cell lysis, extraction with diethyl ether, solid phase extraction and analysis by reverse phase-HPLC was developed, based on a method for the quantification of cADPR [3]. Combined microinjection of ADPR and single cell Ca^{2+} imaging were performed as described [3].

Results: Quantification of APDR by HPLC revealed changes in cellular ADPR levels in human neutrophil granulocytes upon stimulation with fMLP and in Jurkat T-lymphocytes upon stimulation with Concanavalin A (ConA). The basal ADPR level in Jurkat cells was determined to be about 55 pmol/10^6 cells, and a long lasting increase of about 1.5-fold was found upon ConA stimulation. Increase of $[Ca^{2+}]_i$ and activation of TRPM2 by ADPR in Jurkat cells were demonstrated by microinjection with single cell Ca^{2+} imaging and patch clamp experiments. The NAD-glycohydrolase inhibitor Cibacron Blue (3GA) inhibited the increase of intracellular ADPR concentrations upon ConA stimulation and similarly reduced both the peak and the plateau phase of Ca^{2+} influx, as well as the quenching of fura2 fluorescence by Mn^{2+}.

Discussion: The present work demonstrates for the first time the regulation of intracellular ADPR concentrations by extracellular stimuli in two cell systems. Patch clamp and single cell Ca^{2+} imaging data show that physiologically relevant concentrations of ADPR are appropriate to activate endogeneous TRPM2 and to increase $[Ca^{2+}]_i$. Finally, we show that inhibition of ADPR synthesis by 3GA reduced the influx of Ca^{2+} induced by stimulation of Jurkat T cells with ConA. We propose ADPR to be a novel second messenger involved in the complex regulation of $[Ca^{2+}]_i$.

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Keywords: ADP-ribose, TRPM2, Ca^{2+} influx, HPLC, microinjection, patch clamp

EFFECT OF CYCLIC ADP-RIBOSE ON CALCIUM HOMEOSTASIS AND IN VITRO EXPANSION OF HUMAN HEMATOPOIETIC PROGENITORS

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Cyclic ADP-ribose (cADPR) is a universal, potent calcium mobilizer generated from NAD^+ by the ectocellular ADP-ribosyl cyclases CD38 and BST-1. It has been shown to behave as a hemopoietic cytokine, stimulating the in vitro proliferation of committed and uncommitted human hemopoietic progenitors (HP) and also the in vivo expansion of hemopoietic stem cells (1-3). Here, we investigated, (a) the mechanism of influx of cADPR into human HP and (b) the stromal sources of cADPR inducing HP expansion. As to the first task, the enhancing effect of cADPR on colony growth was abrogated by incubation of target cells, unseparated cord blood-derived mononuclear cells (CB MNC) with nucleoside transporter (NT) inhibitors dipyridamole and nitrobenzylthioinosine prior to exposure to cADPR. These results demonstrate that influx of cADPR into human HP is mediated by a concentrative NT. Moreover, we purified from CB MNC $CD34^+$ cells and we investigated a direct effect induced by extracellular cADPR on this cell sub-population. Both the basal $[Ca^{2+}]_i$ and the colony output of $CD34^+$ cells were shown to be increased upon incubation with 100 μM cADPR. The presence of the NT inhibitors dipyridamole and nitrobenzylthioinosine completely abrogated the effect of cADPR on basal calcium and colony growth in this sub-population cell. This indicated a direct effect of the cyclic nucleotide on the $CD34^+$ cells, ruling out the involvement of accessory cells. As to the second task, we generated $BST\text{-}1^+$ and $CD38^+$ stromal cell lines to compare the effect of the long-term culture of CB MNC over these cells. Colony output was highest in CB cells co-cultured over $BST\text{-}1^+$ stroma and was dependent on the extent of BST-1 expression. Our results indicate that extracellular, nanomolar cADPR, as produced by cyclase-positive stroma, is transported into primitive HP via a concentrative NT and improves hemopoietic engraftment in vivo and increases LTC colony output in vitro.

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Keywords: Cyclic ADP-ribose, Calcium, human hematopoietic progenitors, $CD34^+$

CONTROL OF NUCLEAR CALCIUM HOMEOSTASIS OF APLYSIA NEURONS BY MULTIPLE MESSENGERS : ROLES OF NAADP, IP_3 AND cADPR

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Calcium release from intracellular stores in response to hormone or neurotransmitter stimulation is important in the control of synaptic plasticity or gene expression. Accumulative evidences suggest that nuclear calcium plays an important part in these processes. However, the mechanisms involved in the control of nuclear calcium homeostasis remain largely unexplored in neurons. In this study, we investigated the possible role of three intracellular messengers, known to trigger calcium release from the intracellular stores, inositol 1,4,5 trisphosphate, cADPR and NAADP.

The changes of the calcium concentration were assayed in the nuclear envelope as well as in the nucleoplasm using confocal microscopy. Our data showed that addition of IP_3 (10 µM), cADPR (10 µM) and NAADP (500 nM) induced a release of calcium from the nuclear envelope and increased nucleoplasmic calcium concentration. Our data suggest that the NAADP, cADPR and IP_3 receptors are localized preferentially in the inner nuclear membrane. Using a calcium sensitive fluorescent probe, located within the nuclear envelope, we found that the fluorescence intensity increased when ATP (5 mM) was added in the medium bathing the isolated nuclei, indicating the involvement of Ca^{2+} ATPases in the calcium uptake by the envelope. The nuclear calcium pumps were found sensitive to thapsigargin. Depletion of the thapsigargin-sensitive stores significantly reduced the calcium response evoked by IP_3 or NAADP. Interestingly, a NAADP dose-response experiment indicated that addition of nanomolar concentrations (10, 100 and 500 nM) of NAADP resulted in calcium release from every nuclei tested (n=9) whereas addition of micromolar concentrations of NAADP failed to evoke a response in 9 out of 12 nuclei. In those 3 responsive nuclei, the release of calcium was weak compared to the effect of nanomolar concentrations of NAADP. These results are reminiscent of the response obtained in intact cells of the mouse exocrine pancreas in which high concentration of NAADP (50 - 100 µM) does not generate any Ca^{2+} signal suggesting a complete desensitization of the receptor. Our work show that the bell-shape curve of NAADP effect also applies to neurons.

Our data show the first evidence that the nuclear envelope of a neuronal cell is a calcium store sensitive to several intracellular messengers suggesting a multiple control of gene expression.

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Key words: Calcium, NAADP, IP3, cADPR, nucleus, gene expression

FUNCTIONAL SIGNIFICANCE OF S100A4(MTS1) IN TUMOR-STROMA INTERPLAY

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Causal implication of S100A4 in inducing metastases was convincingly shown previously. However, the mechanisms that associate S100A4 with tumor progression are not well understood. As a typical member of the S100 family S100A4 exhibits dual, intra- and extracellular, functions. This work is focused on the extracellular function of S100A4, in particular its involvement in tumor-stroma interplay in VMR tumor cells, which exhibit stroma-dependent metastatic phenotype.

We demonstrated the reciprocal influence of tumor and stromal cells where tumor cells stimulate S100A4 secretion from fibroblasts in culture. In turn the extracellular S100A4 modified the cytoskeleton and focal adhesions and triggers several events in tumor cells. We found stabilization of the tumor suppressor protein p53 and modulation of its function. In particular, extracellular S100A4 downregulates the pro-apoptotic *bax* and angiogenesis inhibitor *thrombospondin-1* gene. For the first time we demonstrate here that the S100A4 protein added to the extracellular space strongly stimulates proteolytic activity of VMR cells. This activity, most likely, is associated with MMPs and in particular with MMP-13. Finally, application of the recombinant S100A4 protein confers stroma-independent metastatic phenotype on VMR tumor cells.

In conclusion, our results indicate that metastasis-inducing S100A4 protein plays a pivotal role in the tumor-stroma environment. S100A4 released either by tumor or stroma cells triggers pro-metastatic cascades in tumor cells.

We will discuss the putative mechanism of S100A4 secretion and pathways of its pro-metastatic influence on tumor cells.

Keywords: S100A4; p53; gene regulation; MMP activation; metastases

S100A1-DEFICIENT MICE DISPLAY CHARACTERISTICS OF HYPERTROPHY AND ENHANCED MYOCARDIAL PERFORMANCE

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S100 proteins are a group of small (10-12 kDa) acidic proteins that undergo a conformational change upon calcium (Ca2+) binding. These proteins have sequence similarity with calmodulin and other calcium Ca2+-binding proteins. Whereas calmodulin functions in a wide range of cell types to transduce the signal of increased intracellular Ca2+, S100 proteins are restricted to specific cell types. Protein S100A1 is mostly abundant in the heart. To understand the in vivo function of S100A1, we have generated S100A1 knock out mice by insertional mutagenesis. The retroviral gene-trap vector inserted in intron 2, disrupting the translation of S100A1 after exon 2. Whereas a transcript encompassing exon 1 and exon 2 can be traced by real-time PCR, northern and western blotting failed to detect S100A1 mRNA and protein, respectively, in any tissue of homozygous mice, confirming the prevalence of S100A1-deficient mice. S100A1-null mice are born at the expected mendelian frequency and appear healthy. Preliminary results indicate that male S100A1 knock out mice have an increased heart/body weight ratio, an indicator of hypertrophy. In addition, male S100A1 knock out mice show enhanced left ventricular systolic pressure, contractility and relaxation under basal conditions and isoproterenol (a beta1- and beta2-adrenergic receptor agonist) stimulation. Real-time PCR revealed a modest increase in beta1-adrenergic receptors and brain natriuretic peptide (BNP), a hypertrophy marker, in both, male and female knock out mice. To confirm our data we will check further hypertrophy markers and perform echocardiography. In an effort to identify molecular pathways altered due to the absence of S100A1 we are comparing gene expression profiles and protein patterns of S100A1-deficient and wild type mice by gene array and 2D-gel electrophoresis, respectively. Our studies provide insight into the role of S100A1 in heart function and possibly disease and may reveal sex-related differences in the handling of S100A1-deficiency.

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C.W. Heizmann, et al. S100 proteins: structure, functions and pathology, Frontiers in Bioscience 7, 2002, d1356-1368, May 1

Keywords : S100A1, heart, hypertrophy, contractility, relaxation, knock out mice

RAGE-DEPENDENT AND RAGE-INDEPENDENT EFFECTS OF S100B ON MICROGLIA

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Besides regulating several intracellular activity, the Ca^{2+}-modulated protein, S100B, exerts regulatory effects on a several cell types once released into the extracellular space or blood (1). S100B is expressed in high abundance and released by astrocytes (1). At the low levels found in the brain extracellular space S100B acts as a trophic factor, protecting neurons against oxidative stress and stimulating neurite outgrowth through its binding to the receptor for advanced glycation end products (RAGE) (2). However, upon accumulation in the brain extracellular space due to increased expression and/or release and/or leakage from damaged astrocytes, S100B might be detrimental to neurons, causing neuronal apoptosis via either RAGE binding (2) or stimulation of nitric oxide (NO) release by astrocytes and microglia (3-5). Microglia are the brain resident macrophages participating in brain inflammatory responses and the pathophysiology of neurodegenerative disorders. In vitro data indicate that at relatively high concentrations S100B can activate microglia via RAGE binding (6) and stimulate NO release by microglia in the presence of lipid A or interferon (IFN)-γ (4,5). We analyzed further the S100B-microglia interaction to get information about the molecular mechanism by which the protein activates microglia. We found that the ability of (relatively high of) S100B to stimulate NO production by BV-2 microglia depended on production of reactive oxygen species and activation of the stress-activated kinases, p38 and SAPK/JNK. However, although these two kinases were shown to be activated by RAGE engagement in other cell types (7), S100B was still able to stimulate NO production by BV-2 microglia overexpressing RAGEΔcyto, a transduction-incompetent mutant of RAGE. Also, in the presence of S100B the amount of NO produced on a per cell basis was similar in RAGE- and RAGEΔcyto-overexpressing BV-2 cells, and it was larger than in control cells suggesting that RAGE transducing activity has no role in S100B-stimulated NO production by microglia, but RAGE extracellular domain is important anyway, probably serving to concentrate S100B on the BV-2 cell surface. S100B has been shown to regulate cell functions independently of RAGE activity (8,9). On the other hand, S100B was able to up-regulate cyclo-oxygenase-2 expression in BV-2 microglia irrespective of the absence or presence of IFN-γ, in a RAGE- and p38-dependent manner. Thus, S100B appears to exert pleiotropic effects on BV-2 microglia, some of which are dependent on RAGE engagement and activity while other effects are independent of RAGE transducing activity.

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Keywords : S100B, microglia, nitric oxide, cyclo-oxygenase-2, RAGE, p38, SAPK/JNK.

CO-DISTRIBUTION OF S100A1 WITH SYNAPSIN I IN DISCRETE BRAIN AREAS: S100A1 INHIBITS THE F-ACTIN BUNDLING ACTIVITY OF SYNAPSIN I

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The Ca^{2+}-sensor protein S100A1 binds several target proteins, and functional correlates of these interactions have been described (1). Recently, S100A1 was shown to bind synapsins I and II in vitro (2). Synapsins I and II are neuronal proteins that anchor synaptic vesicles (SVs) to F-actin in nerve terminals and regulate SV availability for exocytosis in a phosphorylation-dependent fashion (3). The functional significance of S100A1-synapsin interaction is not known at present. While synapsin I and S100A1 co-localize in a neuronal cell line (2), it is not known whether the two proteins co-localize in mature neurons, and whether S100A1 modulates synapsin function. We analyzed possible effects of S100A1 binding to synapsin I. S100A1 caused a dose- and Ca^{2+}-dependent inhibition of the F-actin bundling activity of synapsin I without interfering with synapsin I binding to F-actin or SVs. Also, no effects of S100A1 could be documented on actin polymerization or synapsin I-triggered actin polymerization, and no binding of S100A1 to SVs could be observed. By chemical cross-linking and Western blot analyses, S100A1 was observed to prevent the formation of synapsin I dimers and to form multimolecular complexes in which monomeric synapsin I is associated with dimeric and tetrameric S100A1. An S100A1 mutant lacking the C-terminal extension (mS100A1) also inhibited synapsin I dimer formation and formed complexes of monomeric synapsin plus tetrameric mS100A1. These data suggest that: i) synapsin I dimerization and interaction with S100A1 are mutually exclusive; ii) the C-terminal extension of S100A1 is not required for S100A1 binding to synapsin I; iii) binding of S100A1 to synapsin I inhibits the F-actin bundling activity of synapsin I, without affecting synapsin I binding to either F-actin or SVs; and iv) S100A1 binds to cytoplasmic synapsin I exclusively. Lack of association of S100A1 with SVs and SV-bound synapsin I was documented by Western blot analyses of synapsin I, synaptophysin and S100A1 in brain subcellular fractions. By confocal laser scanning microscopy and immunogold cytochemistry we documented that S100A1 co-distributes with synapsin I in nerve terminals in discrete brain areas. Thus, S100A1 might play a role in the synapsin I-dependent regulation of the actin-based cytoskeleton in nerve terminals; by preventing (cytoplasmic) synapsin I dimerization, S100A1 might be implicated in the regulation of synapsin I-dependent SV clustering, SV attachment to actin filaments and SV availability for exocytosis in the nerve terminal.

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Keywords: S100A1, synapsin I, F-actin, bundling, inhibition, immunocytochemistry.

S100B-INDUCED INCREASE IN PROLIFERATION AND DECREASE IN NGF SENSITIVITY OF PC12 CELLS DEPEND ON PI3-K/Akt (PKB) ACTIVATION

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S100B is a member of a multigenic family of Ca^{2+}-modulated proteins of the EF-hand type with both intracellular and extracellular roles (1). In the nervous system, S100B is expressed in high abundance in astrocytes, oligodendrocytes and Schwann cells, and in several neuronal populations (1). Among the intracellular regulatory roles attributed to S100B is the participation of this protein in cell cycle progression (1). It is long known that the S100B levels are high in tumor cells, compared with normal parental cells (1). However, the molecular mechanism underlying the potential role of S100B in cell proliferation has not been elucidated. Previous work has shown that S100B interacts with the tumor suppressor protein p53, inhibiting its phosphorylation and oligomerization (2), which suggests the possibility that S100B might regulate cell proliferation by blocking the tumor suppressor activity of p53 (4,5). We studied some effects of forced expression of this protein in a neuronal cell line, PC12, which normally does not express S100B mRNA or protein, by the Tet-off technique which allows to modulate the expression of a transfected gene by varying the concentration of doxycycline in the culture medium (6). Expression of S100B resulted in a higher survival under stress conditions and proliferation rate, a smaller extent of apoptosis, and a decrease in sensitivity to the neurite extension activity of NGF (i.e., $S100B^+$ PC12 cells were less able to extend neurite in response to NGF, compared to $S100B^-$ PC12 cells which responded to NGF by extending neurites) (7). These changes were accompanied by increased levels of $p21^{WAF1}$ and increased extent of phosphorylation of the retinoblastoma suppressor protein, Rb, by cyclin D_1-dependent kinase (cdk) 4 (7). We show here that: 1) a larger fraction of cyclin D_1 co-immunoprecipitates with $p21^{WAF1}$ in $S100B^+$ PC12 cells compared with $S100B^-$ PC12 cells; 2) S100B brings about its effects by activating the phosphatidylinositol 3-kinase (PI3-K)/Akt pathway, as higher levels of phosphorylated Akt can be detected in $S100B^+$ PC12 cells than in $S100B^-$ PC12 cells and all the effects of S100B are negated by pretreatment of PC12 cells with the PI3-K inhibitor, LY294002; and 3) the S100B-p53 interaction, that was proposed to mediate S100B-induced cell proliferation in other cell types (5), has no major role in the present case. We propose that S100B stimulates PC12 cell proliferation and reduces PC12 cell responsiveness to NGF by a PI3-K/Akt/$p21^{WAF1}$/cyclin D_1-cdk4/Rb/E2F pathway. These data suggest that S100B may act within cells to promote proliferation and survival and to modulate differentiation. Analyses are in progress to identify the molecular mechanism by which S100B activates PI3-K in PC12 cells.

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Keywords: S100B, proliferation, differentiation, $p21^{WAF1}$, Rb, Akt.

CHARACTERISATION OF MOUSE ANNEXIN A9 GENE STRUCTURE AND PROMOTER FUNCTION

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Annexin A9 is an atypical member of the annexin family of Ca^{2+} and phospholipids binding proteins, firstly identified in EST data bases (Morgan et al.). Its amino acid sequences responsible for calcium coordination are mutated suggesting an atypical, Ca^{2+} independent cellular function in comparison to other family members. In our studies we have cloned the mouse homolog of human annexin A9 and concentrated on the characterisation of its promoter structure and function by application of luciferase gene reporter assays. The mouse annexin A9 consists of 13 exons with exon 1 being non-transcribed. 2.5kb 5´ flanking region containing 5´ non-coding sequence, exon 1 and intron 1 were cloned upstream of the luciferase gene into the pGL3Basic vector and co-expressed in mouse HEPA 1-6 cells with beta-galactosidase encoded by a co-transfected control plasmid. HEPA 1-6 cells were chosen since they express endogenous annexin A9 message as verified by RT-PCR. Further gene reporter assays with series of deletion mutants and primer extention experiments using RLM-RACE revealed the existence of a minimal promoter located at the 3´ end of intron 1. Deletion analysis also identified two further elements within exon 1 with possibly negative regulatory function on gene transcription. This could suggest that the entire exon 1 together with intron 1 is involved in the regulation of mouse annexin A9 transcription.

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Keywords: annexin A9, HEPA1-6, luciferase assay, RLM-RACE

ANNEXIN A2 BINDS SPECIFIC mRNAS AND REGULATES THE TRANSLATION OF ITS COGNATE mRNA

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Annexin A2 (anxA2) is a multifunctional Ca^{2+}- and phospholipid-binding protein. We have previously shown that anxA2 is associated with both active and inactive mRNP complexes (1). However, it is only found in mRNPs or polysomes associated with the cytoskeleton (1, 2). Here we show that anxA2 interacts *in vivo* with c-*myc* and *annexin* A2 mRNAs belonging to this subset of mRNPs, but not β_2-*microglobulin* mRNA translated on membrane-bound polysomes. This supports the assumption that the protein is involved in linking specific mRNPs to the cytoskeleton and thus in their subcellular targeting and/or anchorage. The interaction of anxA2 with its cognate mRNA appears to stabilise the messenger, and *in vitro* translation studies indicate that free anxA2 represses the translation at the level of initiation of its cognate mRNA in a dose-dependent manner. Thus, it is possible that anxA2 by binding to both the 5'- and 3'-ends of mRNAs, generates functional interactions which simultaneously control both the subcellular localisation and initiation of translation of these messengers.

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Keywords: annexin A2, c-myc, mRNA, translation

ROLES FOR ANNEXIN 1 AND ANNEXIN 2 IN POST-ENDOCYTIC SORTING OF EGFR

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Activated EGF receptors (EGFR) are sorted onto internal vesicles of multivesicular endosomes/bodies (MVBs), thus removing receptors from the recycling pathway and targeting them for degradation. Futter *et al.* (1993)(a) identified two major substrates for EGFR tyrosine kinase within MVBs: EGFR itself and annexin 1, and proposed a role for annexin 1 in sorting within the MVB. Annexin 2 has also been implicated in MVB biogenesis (Mayran *et al*. 2003)(b) and in regulating the distribution of recycling endosomes (Zobiack *et al*. 2003)(c). We have investigated the roles of annexins 1 & 2 in MVB biogenesis and receptor sorting within MVBs using gene knockout and RNAi-induced protein depletion. In EGF-stimulated lung fibroblasts isolated from an annexin 1 knockout mouse (d) MVBs formed but the number of internal vesicles per MVB was reduced. Similar results were observed in EGF-stimulated HeLa cells depleted of annexin 1 using RNAi. In contrast depletion of annexin 2 in HeLa cells had no effect on either MVB formation or the formation of internal vesicles within MVBs. Likewise in DT40 cells in which annexin 2 has been deleted MVB formation was unaffected. Thus, the effects of annexin 1 and annexin 2 depletion are distinct with respect to EGFR sorting within MVBs. Inhibition of inward vesiculation in annexin 1 knockout cells was accompanied by a reduced rate of EGFR degradation, although EGF ligand degradation was unaffected. As annexin 1 is a good substrate for the EGFR kinase while annexin 2 is not, it is possible that the effects of annexin 1 on EGFR sorting within MVB are mediated through EGF-stimulated tyrosine phosphorylation of annexin 1. In support of this hypothesis we found that EGF-stimulation caused an increase in inward vesiculation within MVBs and this increase is dependent upon annexin 1.

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IDENTIFICATION OF FUNCTIONAL DOMAINS IN ANNEXIN 11 INVOLVED IN CYTOKINESIS

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Annexins are a family of widely expressed proteins that bind phospholipids in a calcium dependent manner. Annexins contain a homologous C-terminus, containing the annexin core, which is made up of four repeats ~70aa long, these domains are responsible for calcium and phospholipid binding. The amino terminal is unique to each family member, and is thought to confer properties that determine their specific roles in vivo. Annexins have been found to play a role in numerous processes such as vesicle trafficking [1], calcium signalling [2] lipid raft organisation [3] and more recently a role in cell division [4]. Annexin 11 (A11) has been found to re-localise from the nucleoplasm to nuclear membranes during early to mid prophase in A431 and HeLa cells [4]. This nuclear membrane translocation could be stimulated in these cells by treatment with a calcium ionophore. More recently A11 has been found to localise to the midbody during cytokinesis [5]. siRNA was used to knock down A11 expression in A431 cells, this resulted in abnormal cytokinesis and cell death, indicating an essential role for A11 in cytokinesis. siRNA treated cells undergoing cytokinesis remain attached by an abnormal cytoplasmic bridge, a phenotype similar to that observed in cells lacking the mitotic kinesin-like protein, CHO1 [6]. When CHO1 was completely knocked down cells also displayed abnormalities in spindle microtubule bundles, an effect not observed in A11 siRNA treated cells. Systematic analysis of the different domains of the CHO1 protein revealed that the tail region of is important for cytokinesis [7].

The aim of the current studies is to examine which domains of A11 are involved in midbody localisation and therefore play a role in regulation of cytokinesis. Initially investigations will attempt to use A11 fused to GFP to analyse the distribution of the full-length protein, the C- and N- terminals in live and fixed cells during the cell cycle. Previous experiments using siRNA demonstrated the essential role for A11 in cytokinesis, as knockdown of endogenous protein resulted in incomplete cytokinesis and eventual apoptosis. We will rescue this effect through transient transfection of siRNA-hardened A11-GFP fusion into siRNA treated cells. Then through the use of discrete polypeptide sequences of A11 identify the domains of A11 involved in cytokinesis.

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Keywords: Annexin 11, Cytokinesis, Midbody, Kinesin.

EVOLUTIONARY PROFILE OF THE CALCIUM BINDING SITES IN ANNEXIN PROTEINS

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Comprehensive evolutionary analysis of annexin proteins reveals novel insight into their structure-function relationships in 3 critical aspects - membrane phospholipid binding, ion channel activity, and alternative interaction domains. The discovery of ablated and dysfunctional type 2 calcium binding sites in human annexins A9 and A10 [1,2] now extends to analogous representatives that evolved independently in other major phyla, including echinoderms, protostome invertebrates, fungi, plants and protists [3]. Distinct molecular profiles for these unique subfamilies were visualized as signature logos generated from hidden Markov models and as 3-dimensional models portraying the domain architecture. The broad range of structural variation observed in and around annexin type 2 calcium binding sites implied greater functional diversity with respect to their calcium dependency and suggested alternative mechanisms for mediating membrane binding. The regulation of annexin calcium channel activity is purported to reside in a conserved Glu-Arg salt bridge between repeats 2 and 4, but phylogenetic variability in the former site and exceptions to the latter have been identified, as exemplified by the conversion to Gln-Asp in bovine annexin A13. Such findings question the universality and ion selectivity of annexins as atypical calcium channels but provide natural models amenable for empirical testing of revised hypotheses.

Doubts raised about the essential role of calcium in annexin membrane binding and channel activity call for alternative prospects to focus on structural interactions and intrinsic activity. Sequence conservation analysis confirmed the importance of certain aromatic, hydrophobic and charged residues in maintaining alpha-helical folds and tetrad core stability typical of all annexins. It also accentuated the prominence of highly conserved arginine residues in the interloop region between alpha-helices B and C in all 4 repeats that, in contradistinction to the type 2 calcium-binding sites, expose basic positive charges to the concave, cytosolic face of bound annexin molecules. The hypothesis that intrinsic annexin function could involve more specific interactions with cytosolic proteins gained compeling theoretical support from computational, site-rate analyses that identified the concave face as a target of significant evolutionary change in the structural divergence of annexin subfamilies [4].

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Keywords: annexin, bioinformatics, domain modeling, evolution, phylogeny, protein interactions.

STRUCTURAL STUDIES ON PLANT ANNEXINS REVEAL DISTINCT DIFFERENCES TO MAMMALIAN AND VERTEBRATE ANNEXINS

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The crystal structures of annexins from bell pepper and cotton revealed that these proteins also adopt the robust annexin fold, with subtle but significant differences compared to the structures of non-plant annexins.

Calcium-binding has been identified as a landmark feature of animal, plant and metazoan annexin proteins. As structurally established with annexin A5, the canonical type II calcium binding sites are provided by the endonexin sequence, which is disrupted in domains II and III of all plant annexins known to date. The anticipation of dysfunctional calcium binding sites in module II/III of plant annexins is supported by the three-dimensional structures observed crystallographically. In terms of primary and tertiary structures, calcium binding can only be anticipated in domains I and IV. With respect to membrane-binding, it is thus tempting to speculate about a finely balanced interplay between a membrane-anchoring (by aromatic and basic residues on the convex surface) and a bridging mechanism (by calcium ions coordinated by residues of the endonexin sequence).

Plant annexins possess a strictly conserved tryptophan residue in the first domain (IAB loop), which performs a loop motion reminiscent of the one observed with annexin A5. We were able to demonstrate this loop motion by X-ray crystallography for the first time. The IAB tryptophan is hypothesised to play an important role in protein-membrane and protein-protein interactions. A putative RedOx-active motif has been observed with the cotton annexin Gh1, where a unique sulphur cluster is constituted by Met107, Cys111 and Cys238. Even in the absence of a reducing agent, both cysteines exist in their reduced (thiolate-) forms. This MCC motif is a potentially important feature of annexins, since at the level of primary structure it is conserved in several plant annexins as well as annexin A2. The motif is of special importance for the cotton annexin, since it could provide a functional link to RedOx-dependent callose synthase, an enzyme involved in wound response in plants, and oxidative stress response in general.

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Keywords : Annexin, plants, protein crystallography, structural biology

DISCRIMINATIVE SITES IN CONSERVED CORE OF DIFFERENT ANNEXIN SUBFAMILIES OF VERTEBRATES

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The problem of defining specific functions for individual annexins is a recurring theme in annexin research[1]. Last year a method for automated selection of residues that determine the functional specificity of proteins with a common general function was introduced (the specificity determining positions (SDP) prediction method (SDPpred)). Such residues are assumed to be conserved within groups of orthologs (that may be assumed to have the same specificity) and vary between paralogs[2]. Using SDPpred we had found fourteen statistically significant sites in ANX core, which compose distinct pattern for each ANX subfamily. In this study we analyzed all known vertebrate ANXs from Swiss-Prot/TrEMBL database, two additional zebrafish ANXs, ANX3 and ANX13b, which were identified by searching NCBI UniGene gene-oriented clusters of transcript sequences and predicted ANX proteins from pufferfish genome to identify discriminative amino acids in the structure of ANX core. The most of the identified SDPs site in well conserved parts of structural alignment. About half of statistic significant sites display on "membrane" side of protein molecule. Some but not all of these aa take part in known calcium- and phospholipids-binding, as it was shown for ANX1 (ref. 3, 4), so these sites may be determining differences in calcium-dependent ANX-membrane interactions for each subfamily. Other found sites may be determinative for differences in the phospholipids binding, as it was shown for ANX2 and ANX5[5]. C*harge (plus and minus) – neutral – polar* aa substitutions appear in some sites on membrane side of ANXs. Such substitutions may modify membrane binding of ANX as it was shown by Campos et al. for ANX5[6]. Other interesting discriminative aa motif in the cores of different ANXs is *cysteine or methionine – polar – hydrophobic* diversity. Some of these cysteines may be potential targets for chemical modification, e.g. acylation, or have been implicated in oxidative stress response, which was proposed for particular ANXs[7].

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THE VOLTAGE-DEPENDENT ANION CHANNEL MODULATES APOPTOTIC CELL DEATH

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Mitochondria play a central role in energy metabolism, Ca^{2+} signaling, aging and cell death. The voltage-dependent anion channel (VDAC) provides passage for adenine nucleotides, Ca^{2+} and other metabolites into and out of the mitochondria. Recently, we have shown that both ruthenium red (RuR) and the trivalent cation Ru360 induce VDAC channel closure in a time-dependent manner, and stabilize the channel in a completely closed state. Both reagents inhibited opening of the mitochondrial permeability transient pore, while RuR was also shown to inhibit cytochrome *c* release, activation of caspase 3 and apoptotic cell death induced by various stimuli. However, the mechanism(s) underlying the protective action of RuR against apoptosis remains unknown. In this study, the role VDAC in cell death and its interaction with RuR was investigated by over-expression of native and mutated VDAC-1 in the U-937 cell line or yeast. Over-expression of mouse or rat VDAC-1 induced a 3-5-fold increase in apoptotic cell death, from 12% to >70%. This cell death was prevented by preincubation with RuR. As with native VDAC, mutant recombinant VDAC, in which glutamate 72 was replaced by glutamine (E72QVDAC), enhanced apoptotic cell death when expressed in U-937 cells. This cell death was not, however, prevented by RuR. Moreover, neither RuR nor Ru360, while both able to inhibit channel activity of recombinant native VDAC-1 reconstituted into planar lipid bilayer, had any effect on mutated E72QVDAC channel activity. In addition, hexakinase-I interacted with bilayer-reconstituted native VDAC and reduced its conductance, but had no effect on mutated E72QVDAC channel activity. These findings indicate that a single amino acid mutation in VDAC prevents the RuR-mediated protection against apoptosis and provide the first direct evidence for VDAC regulation of mitochondria-mediated apoptotic pathways as well as showing that the protective anti-apoptotic effect of RuR is mediated via its direct interaction with VDAC.

MYELOID RELATED PROTEIN 8 AND 14 INDUCE A SPECIFIC INFLAMMATORY RESPONSE IN MICROVASCULAR ENDOTHELIAL CELLS

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Myeloid related protein (MRP) 8 and MRP14, S100-proteins secreted by activated phagocytes, bind specifically to endothelial cells. The endothelial response to MRP8/MRP14, however, is unknown. Using oligonucleotid microarray analysis we show for the first time that MRP8/MRP14 induce a thrombogenic and inflammatory response in human microvascular endothelia cells by increasing the transcription of proinflammatory chemokines and adhesion molecules and by decreasing the expression of cell junction proteins and molecules involved in monolayer integrity. All changes on gene expression level could be confirmed by biochemical and functional assays. We demonstrate that the expression of MRP8/MRP14 closely correlates with the inflammatory activity in systemic vasculitis confirming their important role for distinct inflammatory reactions in endothelia. MRP8/MRP14 may represent novel targets for anti-inflammatory strategies.

Keywords : S100A8, S100A9, calprotectin, endothelial activation

ASTROCYTIC CALCIUM/ZINC BINDING PROTEIN S100A6 OVEREXPRESSION IN ALZHEIMER'S DISEASE AND IN PS1/APP TRANSGENIC MICE MODEL

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S100A6 (also called calcyclin) belongs to the superfamily of EF-hand calcium-binding protein and is expressed in a tissue-and cell-specific manner. In central nervous system only some regions appears to express S100A6. We have previously expression shown that astrocytic of S100A6 is upregulated in Amyotrophic Lateral Sclerosis (ALS) specifically in brain areas developing neurodegenerative lesions (1, 2). this study we examined S100A6 immunoreactivity (ir) within the temporal neocortex of human sporadic Alzheimer's disease (AD) patients and compared it to two transgenic mouse models for AD. Astrocytes recruitment and activation are a hallmark of many neurodegenerative diseases including Alzheimer's disease (AD). In sporadic AD, S100A6 immunoreactivity was seen both in white and grey matter. In white matter the distribution was large and uniform like GFAP immunoreactivity classically observed during astrogliosis. On the contrary in grey matter, S1006-positive astrocytes were mainly concentrated at the level of the amyloid deposits in senile plaques as shown by Red Congo staining, and double immunolabeling S100A6/Aβ. This pattern was different from S100B distribution which was largely spread in grey matter astrocytes. Likewise in human AD, a strong S100A6-ir was detected in the two transgenic mice models within astrocytes surrounding the amyloid deposits in the grey matter. In conclusion we report here an original expression for S100A6 protein in the grey matter of the neocortex in AD at the level of the amyloid deposit of senile plaques.

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Keywords : Alzheimer's disease - Aβ amyloid – Astrocyte - Calcium - S100 Protein - Zinc

THE CALCIUM-DEPENDENT INTERACTION OF S100B WITH P53 INHIBITS THE FUNCTION OF THE TUMOR SUPPRESSOR

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S100 calcium-binding proteins, such as S100B, are elevated in primary malignant melanoma and are used as markers for this and numerous other cancers.[1,2] Wild-type p53 protein levels are relatively low in these cancer cells (i.e. when compared to cells without S100B), but are elevated when RNA anti-sense to S100B is introduced. This result implicates S100B in the down-regulation of p53 and is consistent with large decreases in p53 protein levels observed previously in transient co-transfections of p53 and S100B[3]. Down-regulation of p53 in primary malignant melanoma cells is likely the result of a direct interaction with S100B, which was observed by co-immunoprecipitation experiments. Furthermore, p53 binds three regions of the S100B promoter, one of which matches the twenty-nucleotide p53-binding consensus DNA sequence perfectly (20/20, 16/20, 17/20). Therefore, when p53 levels increase, it contributes to its own demise by up-regulating the transcription of S100B as part of a feedback loop. This is analogous to what is found for another protein that down-regulates p53, namely hdm2. With the goal of restoring p53 activity, a search for small molecules that bind S100B and prevent S100B-p53 complex formation was undertaken. Chemical databases were computationally searched for potential inhibitors of S100B, and 60 compounds were selected for testing based upon energy scoring, commercial availability, and chemical similarity clustering. Seven of these compounds bound to S100B as determined by steady state fluorescence spectroscopy (1.0 μM $\geq K_D \leq$ 120 μM). Heteronuclear Single Quantum Coherence NMR titrations confirmed binding and indicate that these compounds interact with the p53 binding site on S100B. Additionally, Saturation Transfer Difference (STD) NMR experiments were used to identify protons on the compounds at the small molecule-S100B interface. Models of such inhibitors bound to Ca^{2+}-loaded S100B were calculated using intermolecular NOE data between S100B and the drug, and indicate that these molecules bind into the p53 binding site on S100B defined by helices 3, 4, and loop 2 (termed the hinge region).

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Keywords: S100 proteins, S100B, p53, NMR spectroscopy, Computer Aided Drug Design (CADD).

METAL BINDING AND REDOX MODIFICATIONS OF CYSTEINES AS A COUPLED REGULATION OF S100B PROTEIN

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Redox modifications of cysteine thiols in regulatory proteins are usually interpreted as the formation of intra- or intermolecular disulfide bonds and the impact of such modifications on the structure and function of the modified proteins is discussed. Recently, it has been suggested that the mechanism of cysteine modification is more complicated than simply disulfide bond formation. Cysteine thiols may be, among other modifications, hydroxylated, nitrosylated or glutathionylated *in vivo,* depending on the redox status of the cell. Every such modification may provide a different physiological response [1].

The extracellular activity of the calcium binding protein S100B has been shown to depend on the redox status of the cell. Mutational studies have shown that the presence of cysteine residues is a prerequisite of some of its proper function [2]. It is well known that the activity of S100B strongly depends on its binding of metal ions. In this work we show that calcium, zinc or copper binding specifically influence the reactivity of S100B cysteines toward physiologically relevant, small molecular weight oxidants such as GSNO or GSSG. We show that different cysteine modifications formed impose significantly different effects on protein stability, conformation and target recognition.

S100B provides a copper binding site, which, in the presence of oxygen (ambient air) exhibits absorption spectra similar to those of peroxo-bridged dimers, suggesting that it can activate dioxygen. The energies of transitions of the two characteristic components of these spectra are modulated by the covalent modifications of S100B thiols which are typical for protein redox signal transduction: nitrosylation, glutathionylation and disulfide dimer formation [1]. The copper binding affinities also seem to be affected by these modifications. The mutual influences of the copper binding, oxygen binding and activation, and the thiol redox modifications suggest a regulatory/coupling role for S100B in redox signal transduction and, perhaps, metal trafficking processes.

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Keywords: S100B, nitrosylation, glutathionylation, calcium, zinc, copper

FUNCTIONAL MUTATIONS IN THE C-TERMINAL EF-HAND OF THE CALCIUM BINDING PROTEIN S100A9

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S100A8 (MRP8) and S100A9 (MRP14) belong to the S100 family of calcium binding proteins. Both proteins are specifically expressed in circulating neutrophils and early differentiation stages of monocytes [1], as well as in keratinocytes and epithelial cells under inflammatory conditions [2, 3]. S100A8 and S100A9 are characterized by two calcium binding sites of the EF-hand type with different affinities to bind calcium; a high affinity site at the C-terminus (C-terminal EF-hand; EF-hand II) and a low affinity site at the N-terminus (N-terminal EF-hand; EF-hand I). Like other S100 proteins, S100A8 and S100A9 form complexes, which seem to be the functional form of these proteins. It is known, that formation of S100A8/S100A9 heterodimers is still calcium independent, while formation of heterotetramers occurs only in the presence of calcium [4, 5]. S100A10 (P11) takes a special position within the S100 family because it does not bind calcium due to mutations of two conserved amino acids in the C-terminal EF-hand. Since the formation of noncovalently associated S100A8/S100A9 heterotetramer complexes is known to be calcium dependent, we analysed the role of calcium binding to the C-terminal EF-hand by creating specific S100A9 mutants. In analogy to the C-terminal EF-hand structure of S100A10 we replaced the following amino acids of wild type S100A9 by alanines: asparagine at position 69 (S100A9-N69A), glutamine at position 78 (S100A9-E78A) and a double mutant (S100A9-N69A/E78A). We first analysed the ability of the S100A9 mutants to dimerize with S100A8 in the yeast two-hybrid system and found that heterodimerization is unattached by the C-terminal EF-hand mutations. On the other hand we could show by UV-MALDI-MS and density gradient centrifugation that the S100A9 mutants abolished the formation of S100A8/S100A9 heterotetramer complexes. So we propose that the formation of tetramers is dependent on a functional EF-hand II and that the calcium binding capacity of the S100A9 mutants is decreased. Furthermore the reduced calcium affinity of the mutants shows functional effects, for instance with regard to tubulin polymerization in the presence of S100A8/S100A9. While wild type S100A8/S100A9 complexes promote tubulin polymerization in the presence of calcium, the S100A9 mutants (N69A) and (E78A) showed only a weak polymerization activity.

Thus, we demonstrate for the first time that calcium dependent tetramerformation of S100A8 and S100A9 is a prerequisite for physiological function(s) of these proteins.

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Keywords : S100A8, S100A9, calcium binding, EF-hand

SCREENING PROTECTIVE EFFECTS OF ANTICONVULSIVE DRUGS ON NMDA CHANNELS; COMPARISON OF NERVE GAS COUNTERMEASURE AGENTS

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UV and visible-light fluorescence imaging at single cell resolution is routinely used to measure pharmacological properties of cultured cells. Lower resolution screening techniques are difficult to apply to primary cultures of neural cells from the CNS. Cells tend to cluster and loose adherence to small (coated) glass coverslips. We have developed a technique whereby cells are grown on large coverslips for 1 week or longer and measurements are made in 48 regions on 4 coverslips at a time. Rat cerebellar granule cells were used to measure the effects of drugs on NMDA induced calcium responses.

Procyclidine[1] (similar to Biperiden[1,2,3]) is an anticholinergic drug used in the treatment of Parkinson's disease, dystonia and epileptogenic seizures. Procyclidine also inhibits NMDA receptors and we report effects on NMDA and glycine stimulated responses as well as effects on voltage activated calcium channels. Effects of Procyclidine are compared with those of gacyclidine[4] (GK11) and Huperizine[5]. All three drugs have been recommended as agents in the treatment of nerve gas/organophosphate induced seizures.

Conclusions:
Resolution is enhanced with the new technique since it allows parallel measurements and multiple doses in the same experiment and on a single batch of cells.
Procyclidine and GK11 both protect cells from NMDA induced toxicity whereas no such protective effect was seen for Huperzine. Procyclidine interferes with the GK11 (PCP) binding site.

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Keywords : Procyclidine, Biperiden, Soman, epilepsy, anticholinergics, NMDA

CALCIUM- DEPENDENT NO PRODUCTION IN PANCREATIC ACINAR CELLS

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We have analysed the changes of nitric oxide concentration $[NO]_i$ in acutely isolated pancreatic acinar cells using the fluorescent probes – membrane-permeable 4,5-diaminoflurescein diacetate (DAF-2 DA) and impermeable 3-amino-4-(N-methylamino)-2',7'-difluorofluorescein (DAF-FM). The sensitivity of NO detection upon application of the NO donor spermine-NO complex (sp/NO) or cell stimulation with 10 μM acetylcholine (ACh) was significantly improved if the cell stained with DAF-2 DA were later dialysed using a patch pipette (in whole cell configuration). The sensitivity of NO detection was further increased if the cells were loaded with DAF-FM directly through the patch pipette. The recordings of calcium-dependent chloride currents enabled us to correlate Ca^{2+} signals with the changes in DAF-FM fluorescence. A concentration of 10 μM ACh induced an increase in the fluorescence in the majority of cells loaded with DAF-FM. The cytosolic Ca^{2+} rise preceded an increase in DAF-FM fluorescence. The DAF-FM response was significantly attenuated in cells co-loaded via patch pipette with the NO scavenger iron (II) dithiocarboxysarcosine complex (Fe^{2+}-DTCS) or with reduced glutathione (an abundant cytosolic scavenger of free radicals). Strong Ca^{2+} chelation by millimolar concentration of 2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) in the patch pipette abolished NO responses to supramaximal ACh. Both receptor stimulation by physiological (5 pM) and supramaximal (10 nM) doses of cholecystokinin (CCK) as well as by low doses (50 nM) of ACh triggered an $[NO]_i$ increase. Non-receptor mediated $[Ca^{2+}]_c$ elevation by thapsigargin also resulted in $[NO]_i$ elevation. These results suggest that pancreatic acinar cells can produce NO during physiological activity and when stimulated with pathological doses of secretagogues, and that a cytosolic Ca^{2+} rise is sufficient to induce NO production.

Keywords : nitric oxide, calcium, glutathione, fluorescent NO- sensitive probes

DIFFERENT SPATIOTEMPORAL CALCIUM SIGNALLING IN BLOWFLY SALIVARY GLANDS AFTER SEROTONIN AND HISTAMINE STIMULATION

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Salivary glands of the blowfly, *Calliphora vicina,* are an excellent model for analysing basic mechanisms of cAMP- and Ca^{2+}-mediated signal transduction. The secretion of a KCl-rich saliva is stimulated by the biogenic amine serotonin (5-hydroxytryptamine, 5-HT). 5-HT binds to two different G-protein coupled receptors and causes (1) an increase in intracellular [cAMP] that stimulates transepithelial K^+ transport, and, (2) activation of the phosphoinositide signalling cascade culminating in an increase in intracellular [Ca^{2+}] that activates transepithelial Cl^- transport. High concentrations of 5-HT (> 10 nM) induce global increase in intracellular [Ca^{2+}] while low concentrations of 5-HT (3 nM) induce Ca^{2+} oscillations and propagating intercellular Ca^{2+} waves.

Histamine, another biogenic amine, was suggested to activate only the cAMP pathway, because (1) it produces changes in transepithelial potential (TEP) indicative of cAMP-mediated transepithelial K^+ transport, and, (2) TEP-oscillations without PIP_2 breakdown.
Here we show by microfluorometric Ca^{2+}-imaging using Fura-2: (1) Histamine does cause an increase in intracellular [Ca^{2+}], often exhibiting oscillations with concentration-dependent frequencies. (2) Ca^{2+} elevations are caused by Ca^{2+} release from intracellular stores, shown in Ca^{2+}-free media. (3) The Ca^{2+} elevations are abolished when PLC is blocked by U73122. Histamine-induced Ca^{2+} increase and changes in TEP are also blocked by yohimbine, an antagonist of the 5-HT receptor coupled to the phosphoinositide pathway. In addition, we show that 5-HT- and histamine-induced Ca^{2+} signals have different spatiotemporal patterns: 5-HT produces Ca^{2+} oscillations and propagating intercellular Ca^{2+} waves, while histamine produces local Ca^{2+} oscillations in single cells but never propagating Ca^{2+} waves. At high concentrations of histamine these Ca^{2+} oscillations occur as synchronous Ca^{2+} "beating" in the whole gland.

Supported by DFG Wa 463/9-3

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Keywords : Calliphora vicina, salivary gland, 5-HT, histamine, Fura-2, Ca^{2+} oscillations

A CALCIUM-DEPENDENT PATHWAY IS INVOLVED IN THE FORMATION OF THE EMBRYONIC KIDNEY IN *XENOPUS*

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Morphogenesis of the vertebrate kidney involves the successive formation of the pronephros, the mesonephros and the metanephros. The pronephros is the first to develop and is the functional embryonic kidney in lower vertebrates. Recent studies have implicated a Ca^{2+}-dependent signalling pathway in kidney development, as the loss of function in either of two Ca^{2+}-related proteins is known to result in polycystic kidney disease. The amphibian, Xenopus laevis embryo, is a useful model to study the role of Ca^{2+} signalling in the formation of the pronephros. Indeed, it offers an in vitro system, which consist of ectodermal tissu isolated at blastula stage. These embryonic cells can be induce to differentiate into pronephric tubules, duct and retinoic acid after ectodermic cells were converted into mesoderm by the action of activin A.

In order to study the role played by PKD1 gene and Ca^{2+}-dependent pathways in the process of tubulogenesis (1) we have clone the amphibian orthologue of Human PKD1. Comparison between the deduce sequence of Xenopus polycystin 1 (Xpc1) and the sequence of pc1 from different species revealed a high degree of conservation specially at the level of the transmembrane domains and the COOH terminal part of the protein. The temporal expression pattern of XPKD1 shows that transcripts are detected at all embryonic stages tested, from unfertilised egg (stage 1) to tadpole stage (stage 41). XPKD1 transcripts are also expressed in the presumptive pronephric mesoderm from stage 14 onwards and in animal caps treated with activin A and retinoic acid. (2) We have investigated the role of calcium in this process. i) Pharmacological experiments shows that tubules formation is inhibited by loading the explant cells with the calcium chelator BAPTA. ii) Using the Ca^{2+} sensitive luminescent reporter, aequorin, we have recorded Ca^{2+} transients associated with retinoic acid treatment, before the differentiation of the pronephric tubules. iii) The differentiation of pronephric tubules can be induced by an intracellular Ca^{2+} increase on Activin A treated ectoderm cells.

These data indicate that calcium is a necessary and sufficient signal in the process of tubulogenesis. Using a custom-designed photon-imaging microscope to visualise the Ca^{2+} signalling patterns continually, we are currently in the process of obtaining a spatial and temporal map of the Ca^{2+} signals that occur during the formation of the pronephros in Xenopus laevis embryo. Our work now will be focussed on the role played by polycycstins on calcium entry.

Keywords: Calcium signalling, pronephros, polycystic kidney disease, polycystin, Xenopus

LINKING THE LOCALISATION OF ITR-1 TO ITS CELLULAR AND PHYSIOLOGICAL FUNCTIONS IN *CAENORHABDITIS ELEGANS*

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Calcium is a ubiquitous intracellular signal responsible for controlling a diverse array of processes. The specificity of Ca^{2+} signals is determined, in part, by their spatio temporal pattern, which is achieved using different storage compartments and specialised binding and gating molecules. The inositol 1, 4, 5-trisphosphate (IP_3) receptor (IP_3R) plays a key role in regulating the flux of Ca^{2+} from the endoplasmic reticulum (ER) into the cytoplasm. It follows that the localisation of IP_3Rs may be critical in determining the specificity of Ca^{2+} signals. Our aim is to explore the relationship between IP_3R subcellular localisation and the function of this pathway in the biology of a whole animal. To this end we are establishing a system in *C. elegans* in which IP_3Rs are tagged with GFP. The large size and complex pleiotropic phenotypes associated with the IP_3R make this a challenging goal.

C. elegans IP_3Rs are encoded by a single gene, *itr-1*. Previously the expression pattern of *itr-1* has been identified in transgenic animals expressing truncated forms of ITR-1 fused to GFP (1, 2, 3) and by using anti-ITR-1 antibodies (2). To establish a system that is closer to the 'wild-type' situation we made transgenic animals expressing full-length ITR-1 fused to GFP. This construct was generated using PCR fusion and *in vivo* homologous recombination. To test the ability of the construct to function we used it to rescue the *Sa73* mutant phenotypes, which include disrupted defecation, pharyngeal pumping and fertility. Thus we are able to directly link the localisation of ITR-1 with its functions.

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Keywords: C. elegans, IP_3R, ER, Physiology

THE EFFECT OF MITOCHONDRIAL NETWORK DIVISION ON INTRAORGANELLAR Ca^{2+} WAVES AND Ca^{2+} MEDIATED APOPTOSIS

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The mitochondrial fission factor dynamin related protein-1 (Drp1) is undoubtedly associated with apoptosis induction, but the mechanistic link between mitochondrial structure and vulnerability to apoptosis is still unclear. By transiently or stably overexpressing Drp1 we evaluated the role of mitochondrial network integrity in cellular and organelle Ca^{2+} homeostasis and in apoptotic signalling. 3D digital microscopy revealed fragmented mitochondrial network in Drp1 overexpressing cells, without changes in the endoplasmic reticulum (ER) structure. Agonist dependent cytoplasmic Ca^{2+} signal were unaffected, conversely, single cell imaging of mitochondrial $[Ca^{2+}]$ ($[Ca^{2+}]_m$) revealed propagating intramitochondrial Ca^{2+} waves in the intact but not in Drp1 fragmented mitochondrial network. The blockade of Ca^{2+} waves led to uneven distribution of $[Ca^{2+}]_m$ elevation and to the reduction of the average $[Ca^{2+}]_m$ response as shown by single cell analysis or population measurements, respectively. Accordingly, Drp1 expressing cells showed higher threshold to Ca^{2+} dependent apoptotic stimuli (such as C_2 ceramide), while they were more sensitive to apoptosis induced by staurosporin (which was shown to cause Drp1 dependent redistribution of Bax to mitochondrial membranes). These results highlight the link between mitochondrial 3D structure and the Ca^{2+} signalling patterns of the organelle, and its role in deciphering extracellular stimuli reaching the cell.

S100A16, A NOVEL EF-HAND CALCIUM BINDING PROTEIN HIGHLY EXPRESSED IN MOUSE CNS

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S100 is a multigenic family of Ca2+-modulated proteins of the EF-hand type exclusively expressed in vertebrate and implicated in several intracellular and extracellular functions. We have identified a new member of this family: S100A16. Its mRNA encodes a protein of 103 amino-acids which is highly conserved in mammals and expressed in the brain. Moreover, CAG repeats were identified in the transcribed region which might be associated with triplet repeat disorders of the central nervous system. Our study aims to characterise biochemically the S100A16 protein (secondary structure, Ca2+, Zn2+ and Cu2+ binding) and its distribution and functions in the mouse brain.

ENHANCEMENT OF TRPC3 CHANNEL ACTIVITY BY THE 5-BISTRIFLUOROMETHYL PYRAZOLE DERIVATIVE, BTP2, MAY ACCOUNT FOR BTP2-INDUCED Ca-ENTRY INHIBITION

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BTP2, a 3,5-bistrifluoromethyl pyrazole derivative, has been reported to selectively block calcium release-activated Ca^{2+} entry in T-lymphocytes and Jurkat cells (1,2) that results in inhibition of T-cells proliferation. We examined the effect of BTP2 on receptor operated channel TRPC3, a Ca^{2+} permeable non-selective channel that is DAG activated and store operated in some studies. We used the T3-65 HEK293 cell line with stably over-expressed TRPC3 channels. The channel activity was recorded by patch-clamping in the whole-cell configuration. $[Ca^{2+}]$ measurements were conducted using fura-2-loaded cells. $[Ca^{2+}]$ measurements revealed that SOC activity and TRPC3 activity were both blocked by BTP2 with a similar sensitivity (IC50 approx. 0.3 uM). From patch clamp studies, BTP2 gave a dramatic enhancement of TRPC3 channel activity in response to either OAG or muscarinic receptor activation. The observed TRPC3 current was completely blocked by 10 μM La^{3+}. In contrast, BTP2 did not activate the channel in the absence of agonist or receptor stimulation. The stimulation of TRPC3 channel activity by BTP-2 had an EC50 of approx. 0.5 uM, not significantly different from the IC50 for inhibition of TRPC3-mediated Ca^{2+} entry and comparable with the IC50 of CRAC and SOC-mediated Ca^{2+} entry under similar conditions (1, 2). The data suggest that BTP2 may act by suppression of TRPC3 channel inactivation. TRPC3 activation might be important for the BTP2-dependent Ca-influx inhibition in lymphocytes and other cell types.

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Keywords : TRP channels, store-operated channels, BTP2

CYTOSOLIC CA^{2+} ACTIVATES MITOCHONDRIAL CA^{2+} UPTAKE

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Mitochondria play a significant role in regulating cellular Ca^{2+} homeostasis. The uptake of Ca^{2+} by mitochondria helps to prevent the cytosolic Ca^{2+} concentration from reaching toxic levels following transient elevations (~1 μM) produced by extracellular stimuli, such as hormones, neurotransmitters and growth factors. Recently it has also become apparent that the uptake of Ca^{2+} by mitochondria shapes intracellular Ca^{2+} signals with physiological consequences. The transport of Ca^{2+} into mitochondria occurs via a uniporter driven by the mitochondrial membrane potential. The molecular identity of the uniporter and the factors modulating it are unknown. Studies with isolated mitochondria and patch-clamped mitoplasts estimate the K_D for Ca^{2+} of the uniporter as 10 μM and 19 mM, respectively. Here we show that for intact HeLa cells, mitochondrial Ca^{2+} uptake occurs at cytosolic Ca^{2+} concentrations in the range of 350-400 nM. HeLa cells were loaded with the Ca^{2+} indicator Rhod-2 and mitochondrial Ca^{2+} was measured with confocal microscopy. To investigate whether the kinetics and source of Ca^{2+} affected mitochondrial Ca^{2+} uptake, we induced increases in cytosolic Ca^{2+} by a number of methods including: application of the G-protein-coupled agonist histamine, the sarco-endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin or by inducing Ca^{2+} entry activated by store depletion. We found that regardless of the source or the kinetics of the Ca^{2+} increase, the uptake of mitochondrial Ca^{2+} was activated at much lower cytosolic Ca^{2+} concentrations than previously observed. The sharp inflection observed in mitochondrial Ca^{2+} uptake at cytosolic Ca^{2+} concentrations ~350 nM supports our hypothesis that cytosolic Ca^{2+} activates the uniporter. This finding suggests that for intact cells the mitochondrial Ca^{2+} uptake may be modulated by some factor that is not preserved in the isolated mitochondria or mitoplast preparations.

Keywords : mitochondria, mitochondrial calcium, uniporter

MEMBRANE CONTACT SITES - THE PHYSICAL SUBSTRATE FOR INTRACELLULAR CALCIUM TRAFFIC

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Intra-cellular membrane-bound organelles constantly exchange large amounts of material and information. While most studies focus on traffic between organelles mediated by vesicles, there is another mechanism for intracellular traffic between topologically distinct membranes that does not involve vesicles. Transport of small molecules, in particular calcium ions and lipids, occurs at zones where the endoplasmic reticulum (ER) comes into close apposition with other organelles. Ultrastructural and biochemical studies indicate the existence of stable membrane contact sites, where membranes come close enough to allow proteins on each membrane to form bridging complexes. In recent years, specific components of this short-range communication network have been identified, making this an exciting time to begin to integrate the various different proposed functions of membrane contact sites.

Here, I will review recent advances in our knowledge of two classes of contact site that are involved in calcium traffic: ER–plasma membrane contact sites, and ER–mitochondrial contact sites.

Keywords: lipid binding protein / phospholipid biosynthesis / triad junction / yeast model system

EFFECTS OF SECRETAGOGUES AND BILE ACIDS ON MITOCHONDRIAL MEMBRANE POTENTIAL OF PANCREATIC ACINAR CELLS

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The mitochondrial membrane potential ($\Delta\Psi_m$) is an important parameter, controlling different aspects of mitochondrial metabolism and ultimately ATP production. In this study, the effects of secretagogues and bile acids on the mitochondrial membrane potential of pancreatic acinar cells has been investigated. Measurements of mitochondrial membrane potential were made using the tetramethylrhodamine-based probes TMRE and TMRM.

The first aim of our study was to characterise the relative sensitivities of both the low concentration and the dequench mode of evaluation of changes in $\Delta\Psi_m$. Such a comparative study has not been made in any cell type. The two modes of measurements are based on different principles (Duchen et al., 2003). At low levels of loading, these indicators appeared to have a low sensitivity to the uncoupler carbonyl cyanide m-chlorophenylhydrazone and no response was observed to high doses of the calcium releasing secretagogue cholecystokinin. When loaded at high concentrations, TMRM and TMRE undergo quenching and can be dequenched by mitochondrial depolarisation. Surprisingly, we found the dequench mode to be two orders of magnitude more sensitive than the low concentration mode. Using the dequench mode we resolved mitochondrial depolarisations produced by supramaximal and physiological concentrations of cholecystokinin and also with other calcium-releasing agonists including acetylcholine, JMV-180 and bombesin. Secretin, which employs the cAMP pathway, had no effect on the mitochondrial membrane potential, cAMP was also ineffective. Mitochondrial depolarisations induced by cholecystokinin were abolished by buffering cytosolic calcium. Furthermore, a non agonist dependent calcium elevation induced by thapsigargin, depolarised mitochondria suggesting that a rise in the cytosolic calcium concentration is sufficient to depolarise mitochondria. The final aim of this investigation was to elucidate the effect of putative activators of acute pancreatitis, namely bile acids, on $\Delta\Psi_m$. The bile acids taurolithocholic acid-3 sulphate, taurodeoxycholic acid and taurochenodeoxycholic acid, induced mitochondrial depolarisation, resolved by the dequench mode.

Our experiments demonstrate that physiological concentrations of secretagogues and pathologically relevant concentrations of bile acids trigger mitochondrial depolarisation in pancreatic acinar cells via a calcium dependent mechanism.

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Keywords: Mitochondria, Ca^{2+}, bile, pancreatic acinar cells

A DIFFERENT DISTRIBUTION OF IP3 RECEPTORS IN PANCREATIC AND SUBMANDIBULAR ACINAR CELLS

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Ca^{2+} mobilising agonists evoke different patterns of intracellular calcium signals. These are highly organized and dependent on the distribution of intracellular calcium-release receptors [1]. Pancreatic acinar cells have long been a model for calcium signalling in secretory epithelial cells. When stimulated they characteristically show a calcium signal that is initiated at the apical pole that then spreads as a calcium wave to the basal pole [1]. However, reports in salivary acinar cells indicate that the calcium signal in these cells might originate in the basal not the apical pole [2]. In the present study we tested the idea that maybe the intracellular calcium-release receptors were differently located in the two cell types and this might explain the conflicting data. To this end we immunostained for IP_3 receptor (IP_3R) distribution in mouse pancreatic and mouse submandibular (SM) acinar cells.

Mice were humanely killed and the submandibular and pancreatic glands removed. The glands was then minced (for SM only) and incubated in collagenase (Worthington CLSPA) for 5-10 minutes at 37^{o}C. The tissue was then resuspended in extracellular solution (containing [mM] NaCl 135, KCL 5, $MgCl_2$ 1, $CaCl_2$ 2, Glucose 10, HEPES 10 – pH 7.4 NaOH) and gently triturated to produce a preparation of large clusters of acinar cells (~20 cells). Clusters were then plated on to poly-l-lysine coated coverslips, fixed in methanol (-20^{o}C), and immunostained for IP_3Rs using standard techniques of immunofluorescence. Cell clusters were imaged using confocal microscopy (LSM-510)$_4$ images were then processed using Metamorph (Universal Imaging).

Our results show dramatic differences between the IP3Rs distribution in mouse SM acinar cells compared to mouse pancreatic acinar cells. Our images show that IP_3Rs are restricted to the very tip of the apical pole of pancreatic acinar cells, consistent with previous findings [1]. In contrast, in SM acinar cells IP_3Rs are found in the apical region but also apparently they encircle lateral and basal regions. To define the lumen of the acinar cells, we co-immunostained for ZO-1, a tight junction marker. We found that in both acinar cell types ZO-1 staining exactly overlaid the IP_3R distribution.

In conclusion, our evidence indicates that the IP_3Rs in SM and pancreatic acinar cells are restricted to the acinar lumen. In SM acinar cells the luminal/ductal network is much more extensive leading to an encircling of the cell with IP_3Rs. We suggest that this is likely to lead to the generation of a very different calcium response in SM acinar cells compared to pancreatic acinar cells. This may explain the early reports of basal calcium signals in salivary acinar cells [2]. We are actively trying to measure the calcium response of SM acinar cells.

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Keywords: submandibular salivary glands, Ca^{2+}, IP_3Rs, ZO-1.

IDENTIFICATION OF NOVEL INOSITOL 1,4 5-TRISPHOSPHATE RECEPTOR ($InsP_3R$) INTERACTING PROTEINS

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The $InsP_3R$ is the major intracellular calcium release channel in non-excitable cells. In addition to regulation by $InsP_3$ and calcium it is modulated by post-translational modification and the binding of accessory proteins. This raises the possibility that the $InsP_3R$ acts as a scaffold, anchoring many signalling proteins to the site of calcium release. The aim of our recent work is to identify novel $InsP_3R$ interacting proteins and to determine their effects on $InsP_3$-induced calcium release. Thus, we aim to establish novel links between the $InsP_3$ signalling cascade and other cellular signalling pathways.

To identify proteins associated with the intact $InsP_3R$, avoiding the possible pitfalls of the yeast 2-hybrid system we adopted a proteomic approach. A polyclonal antibody directed against the COOH-terminus of the type 1 $InsP_3R$ was generated and covalently coupled to protein A sepharose. The immobilised $InsP_3R$ antibody was used to immunopurify $InsP_3Rs$ from rat brain lysate which is a rich source of type 1 $InsP_3Rs$. Interacting proteins were eluted from the bound $InsP_3R$ by incubation in low pH buffer. The eluate was subjected to 2D liquid chromatography and mass spec analysis using a ESI-Quadrupole-TOF Mass Spectrometer. Using this technique we have found a number of interacting proteins including Calmodulin which is known to associate with the $InsP_3R$. We are currently confirming the identity of interacting proteins uncovered using this technique by Western blotting of the immunopurified $InsP_3R$ low pH eluate.

POLARISED DISTRIBUTION OF INTRACELLULAR ORGANELLES IN LIVE PANCREATIC ACINAR CELLS

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In this study we have localised the Golgi apparatus in live pancreatic acinar cells and mapped the relationship of this key organelle of the secretory pathway with calcium release sites and other intracellular organelles. We loaded freshly isolated pancreatic acinar cells with organelle specific fluorescent dyes and imaged them using both confocal and Multiphoton microscopy. The Golgi stacks are located immediately between the perigranular mitochondrial belt and the apically localised secretory granules. We will present the complex three dimensional structure of the Golgi stacks recorded in living cells. The ER of the acinar cell is found predominantly in the basolateral part of the cell, with thin strands projecting into the apical region (Gerasimenko et al 2002). The Golgi apparatus is located to the apical side of the bulk of the ER, with the ER projections passing through the Golgi apparatus. We have also characterised the position of the perigranular organelles with respect to the calcium signals in the apical pole of the acinar cell. It was found in electron micrographs and fluorescent imaging that the highest density of mitochondria was along the lateral plasma membrane in the perigranular region. Unlike the rest of the belt mitochondria, these mitochondria are not in contact with the Golgi apparatus. Measurement of Mitochondrial calcium uptake reveals that it is these mitochondria that respond first to physiological calcium signals.

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Keywords : Pancreas, Golgi, Calcium, Mitochondria, Endoplasmic reticulum.

RYANODINE RECEPTORS AND TRANSIENT RECEPTOR POTENTIAL CHANNELS PROVIDE A DISTINCT MECHANISM FOR DEPOLARIZATION OF β-CELLS

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Background and aims: Depolarisation of plasma membrane potential from about -70 mV to about -40 mV is a critical step in the process of stimulation of insulin secretion by glucose and incretin hormones. Closure of the K_{ATP} channels is generally known to mediate such depolarisation. However, closure of the K_{ATP} channels alone cannot account for depolarisation in this range unless there is a depolarising inward current. β-cells have cation channels that belong to the transient receptor potential (TRP) super-family of channels. We investigated whether activation of TRP channels could mediate membrane depolarisation and identified a mechanism that could activate the TRP channels.

Materials and methods: Intracellular free Ca^{2+}concentration ($[Ca^{2+}]_i$) was measured from fura-2 loaded single rat insulinoma cells (S5 cells) derived from INS-1 cells. Membrane potential was measured by perforated-patch whole cell technique.

Results: We first activated ryanodine receptors by 9-methyl, 5,7-dibromo eudistomin D (MBED) and found that this resulted in a prolonged $[Ca^{2+}]_i$ plateau. The $[Ca^{2+}]_i$ disappeared on omission of Ca^{2+} from extracellular medium. This suggests that activation of the RY receptors can cause activation of a group of Ca^{2+} permeable channels. The consequence of activation of the plasma membrane channels on the membrane potential was investigated by patch-clamp technique. Activation of RY receptors depolarised membrane potential from -70 mV to -40 mV. The Ca^{2+}entry across plasma membrane was blocked by membrane depolarisation, SKF 96365, 2-aminoethoxydiphenylborate, $LaCl^{3+}$ and $GdCl^{3+}$, It was not blocked by nimodipine, diazoxide or by treatment with thapsigargin. Replacement of extracellular Na^{+} by choline did not alter Ca^{2+} entry. Ca^{2+} entry deactivated after about five minutes and was activated again in an oscillatory manner.

Conclusion: The properties of the Ca^{2+} entry demonstrated in this study suggest that it is mediated by TRP channels. We demonstrate that such Ca^{2+} entry depolarises membrane potential. Furthermore, we demonstrate that TRP channels can be activated as a consequence of activation of RY receptors.

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Keywords : Ryanodine receptors, TRP channels, beta cells, membrane potential

RNAi REVEALS AN ESSENTIAL ROLE FOR SECRETORY PATHWAY Ca^{2+}-ATPase-1 IN PANCREATIC β-CELL Ca^{2+} HOMEOSTASIS

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Recent studies reveal that the high affinity Ca^{2+}/Mn^{2+} pump involved in secretory pathway Ca^{2+} sequestration, plasma membrane-related Ca^{2+}-ATPase-1 (PMR1/SPCA1), may also be involved in the regulation of Ca^{2+}-entry and $[Ca^{2+}]$ oscillations. Here, we explore the importance of PMR1 in pancreatic islet β-cell Ca^{2+}-homeostasis using a combination of Ca^{2+}-imaging with targeted-aequorins and RNA interference to monitor organelle $[Ca^{2+}]$ changes in β-cells depleted of the Ca^{2+}-ATPase. Endogenous PMR1 mRNA and protein were detected in both isolated rat islets and β-cell derived lines, MIN6 and INS1. Furthermore, subcellular fractionation of the cell lines revealed PMR1 immunoreactivity in both microsomal and dense-core secretory vesicle-enriched fractions. Correspondingly, depletion of endogenous PMR1 with small interfering RNA's inhibited the rate of Ca^{2+}-uptake into the endoplasmic reticulum and secretory vesicles by approximately 20 % in permeabilised INS1 cells. In intact INS1 cells, PMR1 depletion markedly enhanced Ca^{2+}-flux through L-type Ca^{2+}-channels and augmented glucose-stimulated (30 mM glucose), but not basal (3 mM glucose), insulin secretion. Imaging single cells with fluo-3-acetoxymethylester revealed that PMR1 depletion did not affect average cytosolic $[Ca^{2+}]$ increases in response to 30 mM glucose, but appeared to augment insulin secretion by a modification of $[Ca^{2+}]$ oscillation shape. Thus, in response to glucose plus tetraethylammonium, PMR1 depletion transformed rapid $[Ca^{2+}]$ transients into broader $[Ca^{2+}]$ increases on which rapid non-baseline increases were superimposed. Therefore, we reveal that PMR1 plays an important role in the control of β-cell Ca^{2+} homeostasis and insulin secretion. The secretory pathway Ca^{2+}-ATPases, currently consisting of PMR1 and PMR2, also revealed by RT-PCR to be present in β-cells, may play an essential role, which is at least partially non-overlapping with that of SERCAs, in Ca^{2+} homeostasis in secretory and non-secretory cell types.

THE EXPRESSION, ACTIVITY AND LOCALISATION OF THE SECRETORY PATHWAY Ca^{2+}-ATPASE (SPCA1) IN DIFFERENT MAMMALIAN TISSUES

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SPCA1, the mammalian homologue of the *Saccharomyces cerevisiae* protein Pmr1, transports a single Ca^{2+} or Mn^{2+} per ATP hydrolysed into the lumen of the Golgi[1]. The distribution of SPCA1 was investigated at both the mRNA and protein level in a variety of tissues. SPCA1 mRNA and protein were relatively abundant in rat brain, testis and testicular derived cells (myoid cells, germ cells, primary Sertoli cells and TM4 cells; a mouse Sertoli cell line) and epididymal fat pads. Lower levels were found in aorta (rat and porcine), heart, liver, lung and kidney.

SPCA activities from a number of tissues were measured and shown to be particularly high in brain, aorta, heart, fat pads and testis. The proportion of SPCA activity compared to the total Ca^{2+}-ATPase activity was relatively high in brain, aorta, fat pads and testis, suggesting SPCA could play a major role in Ca^{2+} storage within these tissues. The sub-cellular localisation of SPCA1 was shown to be predominantly around the Golgi in both human aortic smooth muscle cells and TM4 cells.

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Keywords: Ca^{2+} Pump, SPCA

EXPRESSION OF SPCA1 ISOFORMS: CELLULAR DISTRIBUTION AND FUNCTION

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The secretory-pathway Ca^{2+} ATPase SPCA1 is a thapsigargin-insensitive intracellular Ca^{2+} (and Mn^{2+}) pump found mostly in the Golgi compartment of cells, and possibly also in more distal components of the secretory pathway (1).

The pump is encoded by the *ATP2C1* gene, which consists of 28 exons and is located on chromosome 3 in human. The *ATP2C1* gene produces at least 4 alternatively spliced transcripts (*ATP2C1a-d*, corresponding proteins SPCA1a-d), which only differ in their carboxy termini (Fig. 1) (2). *ATP2C1a* (SPCA1a, 919 aa) results from splicing of exon 26 to exon 27 and its translational stop codon is located in exon 27; *ATP2C1b* (SPCA1b, 939 aa) arises from splicing of exons 27 to 28 following an activation of internal 5' splice donor site 1; splicing of exon 26 to 28 gives rise to *ATP2C1c* (SPCA1c 888 aa) and activation of splicing at internal site D2 in exon 27 to exon 28 gives rise to *ATP2C1d* (SPCA1d, 949 aa).

First, we developed a common antibody that recognizes all 4 human SPCA1 isoforms. Second, the corresponding cDNAs coding for the 4 isoforms were cloned into mammalian expression vector pMT2 and expressed in HEK and HeLa cells. Their expression was shown by Western blotting and immunocytochemistry and was confined to the Golgi compartment of both cell types. In order to determine the apparent affinity for Ca^{2+} of the 4 isoforms, we are performing Ca^{2+}-dependent Ca^{2+} uptakes with microsomes prepared from SPCA1a-d transfected HEK cells.

Next, we want to further specify the subcellular localization of SPCA1. We are now performing isopycnic sucrose gradient centriguations to discriminate between a localization of SPCA1 in one or more of the classical Golgi compartments (*cis*, *medial*, *trans*), in the TGN, or in both. Finally, we are exploring the role of SPCA1 in post-translational protein modifications (protein processing, glycosylation…) and cell growth by RNA-mediated interference (RNAi). An RNAi vector has already been constructed and its efficiency has been demonstrated by immunocytochemistry (3).

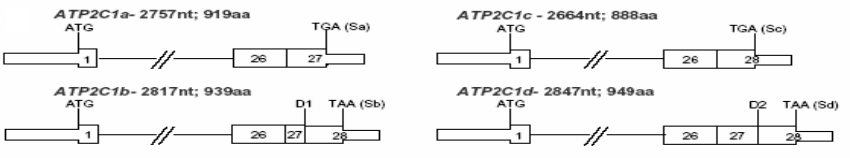

Figure 1. 3' Alternative transcript processing of the human *ATP2C1* gene.

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Keywords: *ATP2C1*, SPCA1, Ca^{2+}, Golgi, RNA interference

IMAGING FERTILIZATION-INDUCED CALCIUM TRANSIENTS IN C. ELEGANS USING MULTIPHOTON MICROSCOPY

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A hallmark for fertilization is a transient increase in the level of free cytosolic calcium, the essential trigger for cell cycle progression. This calcium transient has been observed in a number of species with varying spatiotemporal properties, for example in sea urchin, fish and frogs fertilization induces a single calcium transient, whereas in ascidians and mammals multiple calcium oscillations are observed. Previously in C. elegans Centonze *et al.,* (1997) and Samuel *et al.,* (2001) have shown a cytosolic calcium elevation upon fertilization, but with poor spatial and temporal resolution.

We have studied the spatiotemporal properties of the fertilisation-induced calcium transient in C. elegans using multiphoton microscopy. In the C. elegans hermaphrodite oocytes are formed by budding from a syncytium. As these oocytes move round the gonad arm they undergo maturation, ovulation and are fertilized internally in single file. Since the oocytes can not be removed from the gonad arm we studied the fertilization-induced calcium signal in vivo.
The calcium indicator dye, Fluo-4 dextran (10K) was directly injected into the mature proximal oocyte. The estimated dye concentration in the oocyte is ~10-50μM. The injected worm was subsequently anesthetised, in order to immobilise it for imaging. The oocyte was imaged using multiphoton microscopy.

In C. elegans fertilization induces a single global calcium transient which initiates from the leading edge of the oocyte. The calcium elevation immediately spreads across the oocyte as a wave. The duration of the calcium transient is approximately 10 minutes, after which the cytosolic calcium concentration returns to that preceding fertilization. Directly after the rise in calcium the oocyte passes through the spermatheca and into the uterus.
These results confirm and extend the findings of Centonze *et al.,* (1997) and Samuel *et al.,* (2001).

We intend to develop this technique to image calcium transients in the C. elegans cell cycle to identify the importance of mitotic calcium transients in this species.

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Keywords: calcium, fertilization, C. elegans.

USING A PLCδ PH DOMAIN TO STUDY THE DYNAMICS OF $PtdInsP_2$ AND $InsP_3$ AT FERTILISATION IN SEA URCHIN EGGS

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PH-GFP can be used in living cells as a marker of the $PtdIns(4,5)P_2$-$InsP_3$ signalling pair. However, as competition exists between the two molecules for PH-GFP binding, the distribution of GFP fluorescence must be interpreted carefully.

$InsP_3$, generated by hydrolysis of $PtdIns(4,5)P_2$, plays an important role at fertilisation in all deuterostome species. Recent studies in mouse eggs have paradoxically reported a fertilisation induced increase in $PtdIns(4,5)P_2$ at the plasma membrane, while no change occurs in the cytoplasm.

Here we report that similar studies in sea urchin eggs confirm PH-GFP binding at the plasma membrane, but not at cortical granule membranes in unfertilised eggs. Granule membranes become labelled only when vesicles fuse during exocytosis, causing a net increase in the cortical PH-GFP signal. This is further increased by the growth of microvilli.

PH-GFP binding at the plasma membrane subsequently decreases due to its translocation into the cytoplasm. As the plasma membrane signal falls, the cytoplasmic PH-GFP fluorescence rises to a level comparable to that elicited by microinjection of 10μM $InsP_3$.

A very early and localised increase in PH-GFP signal is also seen at the point of sperm-egg fusion, seconds before the fertilisation calcium wave and onset of cortical granule exocytosis. These data demonstrate that the phosphoinositide signalling pathway acts early in the fertilisation response, and that $InsP_3$ is produced throughout the cytoplasm by the fertilisation wave.

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Keywords : Exocytosis, fertilisation, $InsP_3$, PH-GFP, $PtdIns(4,5)P_2$, sea urchin

ERK ACTIVATION IS REQUIRED FOR S-PHASE ONSET AND CELL CYCLE PROGRESSION AFTER FERTILIZATION IN SEA URCHIN EMBRYOS

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Fertilization of sea urchin eggs results in a large, transient increase in intracellular free calcium concentration ($[Ca_i]$) that is responsible for re-initiation of the cell division cycle. Much of the molecular detail of the mechanisms that underlie resumption of the cell cycle is still unclear. Here we show that activation of ERK1, a calcium-dependent MAP kinase response, is required for both DNA synthesis and cell cycle progression.

We combine experiments on populations of cells with analysis at the single cell level and develop a proxy assay for DNA synthesis in single embryos, using GFP-PCNA. This allows us to compare the effects of low molecular weight inhibitors with a recombinant approach aimed at the same signalling pathway.

We find that inhibition of the ERK pathway at fertilization using either XCL100, a *Xenopus* ERK phosphatase, or U0126, a MEK inhibitor (with its inactive analogue control, U0124) prevents accumulation of GFP-PCNA in the zygote nucleus. We also show that U0126 prevents incorporation of ^{3}[H]-thymidine into DNA. In addition, abrogation of the ERK1 signalling pathway also prevents chromatin decondensation in the sperm pronucleus, nuclear envelope breakdown and formation of a bipolar spindle.

It has previously been reported that a MEK inhibitor, PD98059, triggered DNA synthesis in unfertilized eggs, leading to the suggestion that a decrease in MAP kinase signalling was the trigger for onset of S-phase. We confirm the observation, but not its interpretation. We find that PD98059 induces a very slow increase in GFP-PCNA nuclear accumulation when compared with control embryos, an effect that cannot be mimicked by U0126. We conclude that, physiologically, the onset of S-phase is associated with activation of the ERK pathway at fertilization.

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Keywords : GFP-PCNA, XCL 100, ERK, sea urchin, DNA synthesis

DICTYOSTELIUM CBP3 ASSOCIATES WITH ACTIN CYTOSKELETON AND IS RELATED TO SLUG MOVEMENT

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By employing 2D-PAGE, a protein differentially expressed during the development of *Dictyostelium discoideum* was discovered. The full cDNA of this protein was cloned using RT-PCR technique. The deduced protein is composed of 166 amino acid residues containing four EF-hand domains typical for calcium-binding proteins and was named CBP3. This protein shows little amino acid sequence homology with the other calcium-binding proteins from *D. discoideum* except EF-hand domains. The CBP3 mRNA was absent in vegetative amoebae and accumulated maximally at 6 h of the development on filters. The mRNA level decreased thereafter and disappeared after 12 h of the development, while the protein level peaked at 8 h of the development and was maintained constant thereafter. The mobility of CBP3 on SDS gel was shifted by the treatment of EGTA, confirming the Ca^{2+}-binding activity of the protein.

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Keywords: calcium-binding protein 3; EF-hand; actin cytoskeleton; slug movement; *Dictyostelium discoideum*

CALCIUM DEPENDENT ACTIVATION OF HUMAN NEUTROPHILS FOLLOWING XENOGENEIC CONTACT: THE OCCULT ROLE OF NEUTROPHILS IN XENOGRAFT REJECTION REVEALED

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The role of innate immune cells in the recognition and activation of xenogeneic endothelium has always been considered secondary to the initial insult of xenoreactive natural antibodies (XNA) and Complement. It was argued however, that innate immune cells are capable of recognizing and activating xenogeneic endothelium in the absence of XNA and Complements. Here we show that porcine aortic endothelial cells activate human neutrophils directly. This contact dependent activation causes a transient calcium rise leading to increased reactive oxygen metabolite (ROM) production. Neutrophil gene expression profiling using Adenylate Uridylate-rich Elements (ARE) based microarray revealed a dramatic change in the neutrophil gene profiles upon exposure to PAECs. The PAECs-dependent neutrophil transcriptional activity was further confirmed by real time PCR that revealed a rapid increase in the mRNA level of a number of inflammatory cytokines. The activation of human neutrophils by PAECs was independent of Galα1,3-gal structures since inclusion of saturating concentrations of anti Galα1,3-gal l antibodies had no significant effect. Furthermore this activation was inhibited in the presence of the calcium chelator BAPTA-AM or the reactive oxygen metabolite inhibitor DPI. Our data illustrate the direct activation of innate immune cells by PAECs in the absence of xenoreactive natural antibodies and Complement and suggest alternative recognition sites between PAECs and human innate immune cells.

Keywords : Galα1,3-gal, neutrophils, calcium, ROM, PAECs

ENGAGEMENT OF RAGE ON HUMAN NEUTROPHILS IS LINKED TO CALCIUM PROCESSING AND LEADS TO ABERRANT KILLING OF INGESTED BACTERIA

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Advance glycation end products (AGEs) are a heterogeneous group of non-enzymatically glycated and oxidized species, which occur *in vivo* when proteins and lipids are exposed to aldoses or subject to oxidant stress. Initially, early glycation products (Schiff bases and Amadori products) are reversibly formed whenever plasma glucose levels are elevated. A small proportion of these products undergo further slow irreversible chemical rearrangements to form AGEs, which accumulate in the vasculature under conditions which are accelerated during hyperglycemia and when protein turnover is delayed. AGEs have been found in the tissues and serua of diabetic patients and since diabetes may be also complicated by increased susceptibility to recurrent infection, we investigated the effects of AGEs on human neutrophils, the first cellular line of defense against bacterial infection. We found that human neutrophils expressed receptors to AGEs (RAGE). AGEs bind with high affinity to human neutrophils. The binding was almost completely blocked by excess soluble RAGE, anti RAGE antibodies or antibodies to CML-modified albumin. AGEs induced a dose dependent increase in intracellular free calcium, as well as actin polymerization. Further, AGE-albumin inhibited the transendothelial migration and *Staphylococcus aureus*-induced but not fMLP-induced production of reactive oxygen metabolites. Moreover, although AGE-albumin enhanced neutrophil phagocytosis of *Staphylococcus aureus,* it inhibited bacterial killing as measured by live/dead and blood agar techniques. Our data demonstrate that (i) functional RAGE is present on the plasma membrane of human neutrophils and is linked to Ca^{2+} and actin polymerization, and (ii) engagement of RAGE impairs neutrophil functions.

Keywords: Calcium, AGE, RAGE, phagocytosis, ROM, neutrophils.

ANTIMICROBIAL PEPTIDES FROM SCORPION VENOM INDUCE CA^{2+} RELEASE IN EUKARYOTIC CELLS AT SUBLYTIC CONCENTRATIONS

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Recently bioactive peptides were isolated from the venom of South African scorpions. These peptides were characterized by their antibacterial and antifungal activity at micromolar concentrations (1). Like other host defense peptides they are cysteine-free, positively charged and are able to adopt an amphipathic α helical structure upon association with lipid membranes. In addition we also found that these peptides were able to inhibit superoxide production of granulocytes and to induce degranulation at submicromolar concentrations (2). Parabutoporin (PP, from *Parabuthus schlechteri*) and Opistoporin (OP, from *Opistophtalmus carinatus)* induced reversible Ca^{2+} release from intracellular stores in granulocytes, HL-60 cells (3) and THP1 monocytes. In HL-60 granulocytes we demonstrated that the Ca^{2+} signalling was inhibited by pertussis toxin implying an activation of G-proteins. Whether this activation in HL-60 cells is receptor-mediated or is the result of a direct interaction with a G-protein as suggested for mastoparan is still a controversial issue. The fact that desensitization of the Ca^{2+} transient in HL-60 granulocytes occurred with OP and that the response depends on time of differentiation, favours a receptor-dependent mechanism. No cross-desensitization occurred between OP and the bacterial chemoattractant fMLP indicating the involvement of different types of receptors. Further investigations revealed that the Ca^{2+} release by OP was not mediated via interaction with the FPRL1 receptor, nor by the GPR4 receptor.
In addition these antimicrobial peptides induced Ca^{2+} transients in vascular endothelial cells comparable to signals induced by thrombin and bradykinin.

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Keywords : Scorpion venoms, antimicrobial cationic peptides, Calcium signalling

THE COXSACKIEVIRUS 2B PROTEIN SUPPRESSES APOPTOTIC HOST CELL RESPONSES BY MANIPULATING INTRACELLULAR CALCIUM HOMEOSTASIS

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Enteroviruses, small cytolytic RNA viruses, cause a necrosis-like type of cell death that involves the suppression of an apoptotic program by yet unknown viral anti-apoptotic factors. Using fluorescent calcium indicators and organelle-targeted aequorins, genetically encoded calcium sensors, we provide evidence that the enterovirus 2B protein, a viral membrane-perturbing protein that is localized at ER and Golgi membranes, is responsible for these effects. We found that ectopic expression of the 2B protein causes a decrease in the free calcium content of both the ER and Golgi apparatus. This results both in a decrease in the amount of releasable calcium and, as a consequence, a reduction in the stimulus-dependent Ca^{2+} uptake by the mitochondria, as well as in an increase of Ca^{2+} influx from the extracellular medium. In addition, expression of the 2B protein suppressed cell death induced by certain apoptotic stimuli, ceramide, whose activity its [Ca^{2+}] depending, cycloheximide and actinomycin D. To investigate whether the antiapoptotic activity of 2B is functionally related to its ability to modify intracellular Ca^{2+} fluxes, we characterized two 2B-mutants. We provided evidence that in contrast to the wt 2B protein both mutants are defective in manipulating intracellular Ca^{2+} and for that unable to suppress the cell death induced by apoptotic stimuli as actinomycin D. Taken together, our data indicate that the enterovirus 2B protein is responsible for the viral manipulation of intracellular calcium fluxes and suggest that this ability is an important component of the viral strategy to prevent abortive curtailment of the viral life cycle.

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Keywords : virus, apoptosis, Ca^{2+}, ER, aequorin

DECODING OF Ca^{2+} OSCILLATIONS BY CONVENTIONAL PKCs AT FERTILIZATION

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Ca^{2+} oscillations at fertilization play a major role in the transition from egg to embryo. They are the primary trigger for numerous activation events including resumption of the cell cycle, exocytosis of the cortical granules and entry into the first mitotic division. However, the downstream effectors of this unique Ca^{2+} signal are poorly characterized. In this study, we investigated the possibility that conventional PKCs can decode Ca^{2+} oscillations at fertilization in mouse eggs (Halet *et al.*, 2004).

By monitoring simultaneously $[Ca^{2+}]_i$ and the distribution of GFP-fusion proteins, we demonstrated that PKCalpha and PKCgamma translocate to the plasma membrane of the fertilized egg during each fertilization Ca^{2+} transient. During the first Ca^{2+} transient, cPKC translocation was maximal and proceeded by increments, in phase with the high-frequency Ca^{2+} spikes observed at the top of the transient. Translocation was mediated by the C2 domain of cPKCs, when $[Ca^{2+}]_i$ reaches micromolar levels. Translocation of the GFP-tagged C2 domain occurred selectively during the Ca^{2+} spikes and did not exhibit any incremental pattern, suggesting that another domain of cPKC –possibly the DAG-binding C1 domain- interacts with the plasma membrane and participates in membrane-anchoring of cPKCs.

PKC inhibition with Bisindolylmaleimide I (BIM) inhibited fertilization-induced Ca^{2+} oscillations and cell-cycle resumption. In contrast, PKC stimulation with PMA or overexpression of PKCalpha dramatically increased oscillation frequency. This was correlated with the inhibition or stimulation of store-operated Ca^{2+} entry by BIM or PMA, respectively.

Together, these results demonstrate that cPKCs are major downstream effectors of Ca^{2+} oscillations at fertilization. They are part of a positive feedback loop that sustain the generation of Ca^{2+} oscillations, presumably by promoting Ca^{2+} influx and store refilling.

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Halet G, Tunwell R, Parkinson SJ, Carroll J. (2004) Conventional PKCs regulate the temporal pattern of Ca^{2+} oscillations at fertilization in mouse eggs. J. Cell Biol. 164, 1033-1044.

Keywords: oscillations, fertilization, PKC, influx, GFP

TRYPSIN, PROTEINASES IIA AND XA INDUCE $[CA^{2+}]_i$ SPIKE RESPONSES OR DELAYED OSCILLATIONS IN SAOS-2 OSTEOBLAST-LIKE CELL LINE

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The protease-activated receptor family, which is currently known to consist of 4 members (PAR-1, -2, -3 and –4), belongs to the larger group of G-protein-coupled, seven transmembrane domain receptors. (Trejo J. J Pharmacol Exp Ther (2003), 307: 437-442) Activation of the protease-activated receptors (PARs) occurs through an irreversible proteolytic cleavage of the extracellular domain, resulting on generation of a new N-terminal that functions as a “tethered ligand” by binding intramolecularly to the body of receptor to initiate transmembrane signaling. Coagulant proteases thrombin (factor IIa, FIIa) and factor Xa (FXa) are the physiological activators of PAR-1, -3 and -4 whereas PAR-2 is activated by multiple trypsin-like serine proteases and coagulation factor Xa (FXa) but not by thrombin (FIIa). (Riewald M. Drug Dev Res (2003), 59: 400-407) PAR receptors are expressed on platelets, and a variety of cell types associated with vascular system. PARs expression are also found on sensory nerves, dendritic cells and T cells. Recently, it was demonstrate that human SaOS-2 osteoblast-like cell line expressed PAR-1 and PAR-2 receptors.

The aim of our study was to measure the $[Ca^{2+}]_i$ modification in SaOS cell line by addition of FIIa, trypsin and FXa and determine effects of $[Ca^{2+}]_i$ changes on the cell behaviour. SaOS-2 cells were loaded with Fura-2/AM and intracellular calcium modifications by addition of either FIIa, trypsin or FXa, were measured through 340nm/380nm ratio on fluorescence microscope. Data were recorded and processed with Quanticell 700® software.

FIIa (100nM) induced increase in $[Ca^{2+}]_i$. The calcium spike appeared 10 seconds after addition of FIIa. Addition of trypsin (200nM) induced instantaneous smaller spike response. Finally, FXa induced $[Ca^{2+}]_i$ oscillations. However one minute-delay were necessary to observe calcium oscillations.

These results show that activation of PARs receptor gives rise to $[Ca^{2+}]_i$ changes in SaOS-2 cell line. The understanding of delay for $[Ca^{2+}]_i$ oscillations generation, also observed on endothelial cells (J. Heemskerk, data not published) is the next step in our study.

DEPENDENCE OF THE RATE OF PHOSPHORYLATION OF MYOSIN LIGHT CHAINS-2 ON THE CONCENTRATION OF Ca IONS

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The phosphorylation of skeletal and heart muscle myosin light chains, although isn't an obligatory condition for interaction of actin and myosin, has a regulatory action for this process. The myosin light chains of heart and skeletal muscle of rabbit was phosphorylated by light chain myosin kinase. It was revealed that this process depends on concentration of Ca ions and ionic strength of solution. The total content of myosin light chains-2 in the presence of myosin light chains kinase is decreased for sure from 37,6 to 26,6 rel% with 0,05 mM Ca^{2+} and to 22,6 rel% with 0,1 mM Ca^{2+}. What about phosphorylated myosin light chains-2 content in the presence of corresponding kinase it increases from 12,1 rel% to 22,7 rel% with 0,05 mM Ca^{2+} and to 30,1 rel% with 0,1 mM Ca^{2+}. Simultaneously it was shown that the Ca-ATP-ase activity of myosin in the presence of myosin light chains kinase increased 1,3 times as much. There have been revealed a straight dependence of this activity on the level of phosphorylation of light chains myosin. The kinetics of Ca-ATP-ase reaction of myosin reflected its sigmoid character, which testifies the cooperative mood of the enzymatic reaction. It may be assumed that alterations in the rate of phosphorylation of myosin light chains has a definite influence on the activation of myosin by Ca ions. This phenomenon will undoubtedly be reflected on the actin-myosin interaction and the whole function of contractile apparatus of skeletal and heart muscle.

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Keywords: myosin light chains, phosphorylation, Ca ions

MOLECULAR CHARACTERIZATION OF THE TUFTELIN GENE IN THE MOUSE BRAIN AND IN PC12 AND NEUROBLASTOMA

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The tuftein gene is expressed already at very early stages of tooth bud development. It is thought to play an important role during the development and mineralization of enamel. Our search of the EST database and our previous studies using immunohistochemistry, in-situ hybridization, confocal microscopy, Western blot analysis and DNA sequencing, have shown that the tuftelin **mRNA and protein** are also expressed in various normal and cancerous soft tissues, such as embryonic morula, embryonic stem cells, breast, kidney, lung, eye as well as brain (specifically in neurons) and peripheral nerves (Mao et al. 2001, Deutsch et al. 2002, Leiser et al. 2002). These data suggest that tuftelin may have a universal role and/or is multifunctional.

The aim of the present study is to quantify and characterize the distribution, age dependent expression, and differential expression of tuftelin mRNA in the mouse brain, in different regions of the brain, and in specific cell line models of the brain such as PC12 and neuroblastoma.

Methods: Quantification of the tuftelin mRNA was performed using the real-time quantitative PCR (LightCycler, Roche) of total RNA from 10 different regions of the mouse brain (Chemicon biotest). Alternative mRNA splicing of tuftelin in the mouse brain and in the different cell lines were studied by RT-PCR of the total RNA followed by cloning into pGEM vector and DNA sequencing.

Results: Real-time quantitative PCR showed that the level of tuftelin mRNA expression in the brain increases significantly with age from pre-natal to 7-month-old Balb\C mice ($p<0.03$). Tuftelin expression was tested in 10 different brain areas and the expression was highest in the cerebellum, striatum and mid brain. Two different tuftelin mRNA isoforms were detected in the mouse brain; the first contained all 13 exons and the second lacked exon 2. Both mRNA transcripts contained a single nucleotide substitution: 590 C to T (compared with MacDougall et al., 1998). This single nucleotide substitution gives rise to a single amino acid substitution (histidine to tyrosine) in the predicted amino acid sequence of the tuftelin protein. The lattar may represent a single nucleotide polymorphism or a mutation that may give rise to a phenotype. DNA sequencing reveled that the tuftelin isoform lacking exon 2 is expressed in the rat PC12 cell line. In the human neuroblastoma cell line 2 different isoforms were found, the first lacked exon 2 and the second lacked exons 2 and 3.

Conclusion: The present results and ongoing structural and functional studies will contribute to the understanding of the role of tuftelin in the normal, aging and diseased brain.

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Key words: Tuftelin, Real-time PCR, PC12, Splicing, Brain, Neuroblastoma.

RECOVERIN AND RHODOPSIN KINASE ACTIVITY IN DETERGENT-RESISTANT MEMBRANE RAFTS FROM ROD OUTER SEGMENTS

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Detergent resistant membranes (DRM) or lipid rafts have been isolated from bovine rod outer segments and are known to contain several signalling proteins including the heterotrimeric G-protein transducin and its effector cGMP-phosphodiesterase PDE6 (1-5). We report here the presence of rhodopsin kinase and the neuronal calcium sensor recoverin in DRM that were isolated either in light or in the dark at high and low Ca^{2+}-concentration. Inhibition of rhodopsin kinase activity by recoverin was more effective in DRM than in the initial rod outer segment membranes. The Ca^{2+}-sensitivity of rhodopsin kinase inhibition in DRM was shifted to lower free Ca^{2+}-concentration with an IC_{50} of 0.76 μM, which is in the physiological range of free Ca^{2+} in rod cells. We relate this effect to the high cholesterol content of DRM, since manipulating the cholesterol content of rod outer segment membranes by methyl-β-cyclodextrin yielded a similar shift of the Ca^{2+}-dependent dose-response curve of rhodopsin kinase inhibition. Furthermore, a high cholesterol content in membranes also increased the ratio of membrane bound to cytoplasmic free recoverin. These data suggest that the Ca^{2+}-dependent feedback loop that involves recoverin is spatially heterogeneous in the rod cell.

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Keywords: recoverin, rhodopsin kinase, lipid rafts, termination of phototransduction

POSSIBLE ROLE OF TRPC CHANNELS FOR INSULIN-MEDIATED GLUCOSE UPTAKE IN SKELETAL MUSCLE

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The involvement of Ca^{2+} in insulin-mediated glucose uptake by skeletal muscle is a matter of debate. We have now studied a possible role of Ca^{2+} influx via canonical transient receptor potential (TRPC) channels in insulin action. Pharmacological inhibition of TRPC channel activity decreased the rate of Ca^{2+} influx as well as the insulin-mediated glucose uptake in mammalian limb muscles. Basal, hypoxia- and contraction-mediated glucose uptake were not affected by TRPC inhibitors. Manoeuvres that increased Ca^{2+} influx also increased insulin-mediated glucose uptake both in normal and diabetic *ob*/*ob* muscles. TRPC6 expression (mRNA and protein) was higher in *ob*/*ob* than in control muscles Thus, changes in the Ca^{2+} influx via TRPC channels have large effects on the insulin-mediated glucose uptake and hence these channels provide a novel target for therapeutic interventions in insulin resistant conditions.

Keywords: TRPC, insulin, Ca2+, diabetes

ACTIVITY-DEPENDENT CYTOTOXICITY AND INCREASED PROLIFERATION IN CELL LINES TRANSFECTED WITH TRPV2 CHANNEL

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TRPV2 (GRC, VRL-1) is a calcium permeable cationic ion channel belonging to the Vanilloid subfamily of transient receptor potential (TRP) channels. Studies have shown that upon stimulation of cells by growth factors, TRPV2 translocates from intracellular pools to the plasma membrane *via* a PI-3 kinase pathway, leading to a small but sustained calcium influx. To study membrane expression of TRPV2 channel by immunofluorescence, we have introduced a Flag or HA epitope in the first extracellular loop of mouse and human TRPV2 channel, respectively.

Overexpression of the mTRPV2-flag channel in HEK293 cells grown in standard culture medium (10% FCS) lead to morphologic alterations and cytotoxicity. These toxic effects were abolished by serum starvation, inhibition of PI-3 Kinase by LY294002 and low extracellular calcium concentrations. A mTRPV2-flag channel carrying a charge substitution (E→K) in the pore-forming domain (mTRPV2$_{E594K}$-flag) showed no cytotoxicity although the protein was expressed at the plasma membrane. Interestingly, theses toxic effects were not observed when mTRPV2-flag was expressed in CHO cells. Similar results were found with the human TRPV2 channel: an activity-dependent cytotoxicity was observed when the human channel was expressed in CHO but not in HEK cells. The introduction of a charge substitution in the pore region of hTRPV2-HA (hTRPV2$_{E599K}$-HA) also abolished cytotoxicity when expressed in CHO cells. Thus, expression of TRPV2 in cell lines induces cytotoxicity that is both activity- and species-dependent. Cytotoxic effects seem to be mediated by a constitutive channel activity likely due to the lack of a specific regulation system.

In order to identify mechanisms that regulate TRPV2 channel activity, we have established monoclonal CHO cell lines stably transfected with mTRPV2 cDNA. These cells lines showed a higher proliferation rate than parental cell line. In addition, we showed that after a six hour serum starvation, TRPV2 expressing cells presented enhanced calcium oscillations upon serum re-introduction. Our preliminary data suggest that serum induces translocation of the channel from intracellular compartments to the plasma membrane. We are currently investigating pharmacology and molecular determinants of TRPV2 translocation and activity.

Keywords : TRP channel, cytotoxicity, proliferation, trafficking, calcium influx.

DIFFERENTIAL ROLE OF TRP CHANNELS IN CA^{2+} ENTRY AND PROLIFERATION OF PRIMARY HUMAN PROSTATE CANCER EPITHELIAL CELLS

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One of the major clinical problems in prostate cancer is the remarkable cells ability to survive androgen withdrawal. Unknown mechanisms must underlie this androgen-independent survival and thus represent an attractive therapeutic option. Here, we show that cytosolic Ca^{2+} oscillations produced by alpha1-adrenoceptors (alpha1-AR) stimulation needed TRPC6 gating to mediate the mitogenic effect of catecholamines on the prostate growth *via* the Ca^{2+}-dependent transcription factor – nuclear factor of activated T cells (NFAT) –. A decrease of endogenous TRPC6 expression induced a loss of the α1-agonist-mediated Ca^{2+} entry and the associated proliferating effect. Moreover, NFAT translocation is enhanced in alpha1-agonist-treated human prostate cancer epithelial (hPCE) cells. In contrast, extracellular ATP-mediated Ca^{2+} entry that induces a growth arrest did not require TRPC6 and failed to activate NFAT signalling pathway. Thus, by modulating TRPC6 expression, aberrant alpha1-ARs-agonists-promoting hPCE cells proliferation can be reduced, a finding directly related to the clinical progression of prostate cancer.

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Key words: prostate cancer, α1 adrenoreceptor, Ca^{2+} entry, TRPC6, cell proliferation

CONCENTRATIVE INFLUX OF FUNCTIONALLY ACTIVE CYCLIC ADP-RIBOSE IN GRANULOCYTE-DIFFERENTIATED HL-60 CELLS

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Native human HL-60 cells do not express CD38, a multifunctional ectoenzyme which generates cyclic ADP-ribose (cADPR), a potent calcium mobilizer. However, when they are induced to differentiate to granulocytes by treatment with retinoic acid (RA), HL-60 cells express CD38 and accumulate cADPR, both processes playing a causal role in RA-induced differentiation. Other granulocyte differentiation-inducers, including dimethylsulfoxide (DMSO), fail to express CD38. Here, we investigated whether treatment with DMSO involves any changes in the cADPR/intracellular calcium ($[Ca^{2+}]_i$) signalling system in HL-60 cells. Specifically, we investigated whether DMS0 affects those nucleoside transporters (NT), both equilibrative (ENT) and concentrative (CNT), recently shown to mediate influx of extracellular cADPR. Semi-quantitative polymerase chain reaction analysis of transcripts, binding of [^{3}H] nitrobenzylthioinosine (NBMPR) to intact cells, and influx experiments of extracellular cADPR (with selective inhibitors of NT as NBMPR or in specific conditions) were comparatively performed in uninduced and in DMSO-differentiated HL-60 cells. The control cells showed uptake of cADPR across ENT2, while influx of cADPR into the DMSO-induced cells occurred mostly by concentrative processes mediated by CNT3 and by a NBMPR-inhibitable concentrative NT previously designated cs-csg. DMSO-differentiated, but not native HL-60 cells, accumulated cADPR and showed increased $[Ca^{2+}]_i$ levels when grown in a transwell co-culture setting over CD38-transfected 3T3 fibroblasts where nanomolar cADPR concentrations are present in the medium. NBMPR inhibited both responses of DMSO-induced cells. Thus, concentrative influx of extracellular cADPR across CNT3 and cs-csg NT could surrogate absence of CD38 in eliciting cADPR-dependent $[Ca^{2+}]_i$ increases in granulocyte-differentiated HL-60 cells or in other CD38$^-$ cells.

IMAGING NAADP-MEDIATED CA^{2+} SIGNALING

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Introduction: Nicotinic acid adenine dinucleotide phosphate (NAADP) is the most potent Ca^{2+} mobilizing compound known so far. In human T-lymphocytes NAADP evokes Ca^{2+} signalling at concentrations in the low nanomolar range (10 to 100 nM) whereas micromolar concentrations of the Ca^{2+} mobilizing messengers IP_3 and cADPR were required [1]. Importantly, an active NAADP/Ca^{2+} signalling system is essential for T cell Ca^{2+} signalling [1]. Here, we report on the characterization of subcellular and global Ca^{2+} signals upon microinjection of NAADP.

Materials and methods: Conventional Ca^{2+} imaging was performed in fura2-loaded human Jurkat T cells as described [2,3]. In brief, we used an Improvision imaging system (Tübingen, Germany) consisting of a monochromator system (Polychromator IV, TILL Photonics, Graefelfing, Germany) and a gray-scale CCD camera (type C4742-95-12ER; Hamamatsu, Enfield, United Kingdom; operated in 8-bit mode) built around a Leica DM IRB2 microscope at 100-fold magnification. Confocal images were constructed by mathematical deconvolution based on the point-spread function using a no-neighbor algorithm (Openlab software, Improvision).

Results and Discussion: Upon microinjection of NAADP local Ca^{2+} signals of low amplitude and small diameter were detected. Global NAADP-mediated Ca^{2+} signals were independent of blockade of the IP_3 receptor by heparin, but were sensitive to co-injection of the ryanodine receptor antagonist ruthenium red [4]. This pharmacological approach was confirmed using T cell clones stably transfected with plasmids expressing antisense mRNA targeted specifically against ryanodine receptors [4,5]. The central findings of our study are: (i) local NAADP-dependent Ca^{2+} signals were resolved by rapid confocal Ca^{2+} imaging, and (ii) ryanodine receptors are the major intracellular Ca^{2+} release channels involved in NAADP induced global Ca^{2+} signals. The latter finding is in agreement with data from additional higher eukaryotic cell systems [6], whereas in lower eukaryotic cells, e.g. the sea urchin egg, a separate NAADP-sensitive Ca^{2+} release system appears to be expressed [7].

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Keywords: NAADP, calcium signalling, confocal imaging, T-lymphocytes, ryanodine receptor

FUNCTIONAL INTERACTION OF cADPR- AND IP_3-INDUCED Ca^{2+} SIGNALS IN 3T3 FIBROBLASTS STIMULATED VIA PURINERGIC P2Y RECEPTORS

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Native murine fibroblasts generate Ca^{2+} signals following stimulation of P2Y purinergic receptors and due to activation of the IP_3 cascade (1). Conversely, native 3T3 fibroblasts failed to generate cyclic ADP-ribose (cADPR), another Ca^{2+}-releasing metabolite, although being intrinsically responsive to it (2). The mechanisms of cADPR-induced Ca^{2+} responses and of their interaction with the IP_3 system were investigated in 3T3 fibroblasts, (i) transfected to express the ADP-ribosyl cyclase CD38, (ii) transfected with the antisense CD38 cDNA, as control, (iii) pre-loaded with cADPR itself by incubation with extracellular cADPR. Intracellular cADPR content in $CD38^+$ and in cADPR-pre-loaded 3T3 cells was 50.5±8.0 and 42.3±5.2 pmol/mg protein, respectively, as measured by an enzymatic cycling assay (3). P2Y stimulation with 100 μM ATP on FURA2-AM-loaded $CD38^-$ cells yielded a low increase of intracellular Ca^{2+} ($[Ca^{2+}]_i$) and a much higher Ca^{2+} signal both in CD38-transfected and in cADPR-pre-loaded cells. The use of 8-Br-cADPR, an antagonist of cADPR, significantly reduced the ATP-induced calcium response in $CD38^+$ 3T3 cells, clearly indicating a role for cADPR in the $[Ca^{2+}]_i$ increase. cADPR induced the propagation of localized, IP_3-dependent pacemaker Ca^{2+} signals from the plasmamembrane toward the internal environment, thereby generating a global Ca^{2+} wave affecting the whole cell. Therefore, in 3T3 fibroblasts, IP_3- and cADPR-specific, independent receptors can functionally cooperate by mechanisms involving IP_3-initiated low Ca^{2+} signals and subsequent cADPR-dependent propagation and amplification to generate a global Ca^{2+} response.

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Keywords: cyclic ADP-ribose; Ca^{2+} signaling; 3T3 cell; CD38; signal transduction.

P2Y-RECEPTOR MEDIATED SIGNALLING IN HUMAN SKELETAL MUSCLE BY EXTRACELLULAR ATP

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ATP is an important neurotransmitter in the central nervous system. In skeletal muscle, ATP is released at the neuromuscular junction and has a postulated role in various regulatory processes including cell proliferation, muscle contraction and differentiation (1).

We have investigated the role of extracellular ATP on primary human skeletal muscle cells under inhibitory conditions of extracellular nucleotide hydrolysis. ATP was capable to trigger Ca^{2+} transients via P2Y receptors while Ca^{2+} influx via P2X receptors could not be observed. ATP propogated the inositol phosphate (IP) production with an EC_{50} of 21.3 µM. The phospholipase C (PLC) inhibitor, U73122 completely abrogated Ca^{2+} transients and IP-formation. The nonselective P2Y receptor antagonists, suramin and reactive blue 2 also inhibited the formation of IP by 94.8% and 95.3% respectively. The P2Y1 selective antagonist, A3P5P blocked the ATP stimulated IP production by only 38.9% indicating the additional participation of the P2Y2 receptor.

The role of ATP as a neurotransmitter makes it conceivable to search for an exercise induced plasticity signal in skeletal muscle cells. The activation of extracellular signal-regulated kinase (ERK) 1/2 has been linked to exercise in skeletal muscle (2).

Interestingly, extracellular ATP triggers ERK 1/2 phosphorylation in a PLC dependent manner. Suramin and reactive blue 2 abolished ERK 1/2 activation while P2Y1 receptor antagonists only had partial inhibitory effects. The MEK inhibitor PD98059 also completely inhibited the ATP mediated ERK 1/2 phosphorylation. The chelation of intracellular Ca^{2+} or IP_3 receptor inhibitor, Xestospongin C, had no influence on ERK 1/2 activation. Similar observations were obtained with protein kinase C (PKC) inhibitors, GF 109203X and chelerythrine. Taken together, these results show that extracellular ATP phosphorylates ERK 1/2 in a PLC dependent and a Ca^{2+} - PKC-independent manner in differentiated human skeletal muscle cells, highlighting its possible role in muscle plasticity.

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TIME SENSING BY NAADP RECEPTORS

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Nicotinic acid adenine dinucleotide phosphate (NAADP) has recently been identified as a novel intracellular Ca^{2+} mobilising messenger[1]. In the sea urchin egg, it seems that the NAADP activated Ca^{2+} channel is expressed on a novel calcium store distinct from the endoplasmic reticulum[2]. Very little, however, is known concerning the biochemical properties of the NAADP-binding protein. Radioligand experiments have demonstrated that NAADP binds to the receptor irreversibly. The NAADP receptor can therefore be labelled in its native membrane, solubilised and recovered as a high molecular weight protein following gel filtration[3]. In the present study we describe a slow stabilisation process that takes several minutes to develop and is dependent upon the time the receptor is exposed to its ligand.

Sea urchin (*Lytechinus pictus*) egg homogenates were prepared as previously described[3]. Homogenates were incubated with [^{32}P]NAADP in an intracellular-like medium and unbound [^{32}P]NAADP was removed by centrifugation. Homogenates were solubilised in Triton X-100 (1% w/v)[3], recovered by centrifugation and resolved on a gel filtration column.

Analysis of labelled receptors fractionated by gel filtration revealed that receptor-ligand complexes were less prone to dissociation when incubated with radioligand for longer durations. For example, radioactivity recovered in the peak fraction (corresponding to bound radiolabel) was 15 ± 1 % and 32 ± 2 % of the total radioactivity injected following labelling for 5 and 60 mins respectively. This clear time-dependence was remarkable as exposure to ligand was performed several hours prior to fractionation by gel filtration. However, when these same receptor-ligand complexes were resolved in the presence of phosphatidylcholine (1mg/ml), time dependent dissociation was prevented (28 ± 3 and 34 ± 1 %, 5 and 60 mins respectively). Intriguingly, though receptor-ligand complexes were less stable when labelled for shorter durations, the extent of labelling observed was no different at 5 (2.0 ± 0.3 fmol/µl) and 60 mins (2.5 ± 0.3 fmol/µl). This suggests that the rate of receptor stabilisation is slower than the rate of receptor-ligand association.

Upon binding their ligand, NAADP receptors undergo a slow stabilisation process, dependent on the time receptors are exposed to their ligand. This property endows the NAADP receptor with the extraordinary ability to detect the duration of its activation.

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Keywords: NAADP, Calcium, Sea Urchin, Gel filtration, receptor, phosphatidylcholine.

UNUSUAL ADENYLIC DINUCLEOTIDES PRODUCED BY ADP-RIBOSYL CYCLASES: EFFECTS ON CALCIUM HOMEOSTASIS IN 3T3 FIBROBLAST

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The ADP-ribosyl cyclases from Homo sapiens (CD38, recombinant), from the mollusc Aplysia californica (recombinant) and from the sponge Axinella polypoides (purified to homogeneity) are able to produce three hitherto unknown nucleotides (P18, P24 and P31, from their HPLC retention times) from the powerful intracellular calcium mobilizer cyclic ADP-ribose (cADPR) and adenine. By mass spectrometry of the products of their enzymatic digestion, P31 was identified as diadenosine 5',5'''-P1,P2-diphosphate (Ap2A) and P18 and P24 as two isomers thereof, where one of the adenine moieties is bound to the C1 of the ribose via a nitrogen atom different from the "usual" N9. NMR analyses demonstrated that in P24 the unusual N-glycosidic bond involves the N3 atom of adenine while in P18 spectral analyses suggest involvement of N1. 3T3 fibroblasts transfected with the sense and antisense cDNA for human CD38 ($CD38^{pos}$ and $CD38^{neg}$, respectively) were used as target cells to explore the effects of these dinucleotides on intracellular calcium. $CD38^{pos}$ 3T3 produce endogenous cADPR and thus have a higher basal $[Ca^{2+}]_i$ compared to $CD38^{neg}$ 3T3 (1). Ap2A has been recently described as a modulator of the ryanodine/cADPR-gated Ca^{2+} release channel in rat brain and skeletal cardiac muscle (2). Indeed, incubation with 10 μM Ap2A for 18 h induced a higher increase of the $[Ca^{2+}]_i$ in $CD38^{pos}$ as compared to $CD38^{neg}$ 3T3 (2.2 ± 0.2 vs. 1.6 ± 0.1 fold increase over untreated controls, respectively). Conversely, P18 (1 μM for 18h) induced a 50% decrease of basal $[Ca^{2+}]_i$ in both $CD38^{pos}$ and $CD38^{neg}$ 3T3: subsequent addition of thapsigargin released a two-fold higher amount of calcium from P18-treated cells compared to untreated controls, indicating an increased calcium storage content. Finally, exposure of $CD38^{pos}$ and $CD38^{neg}$ 3T3 to 1 μM P24 for 18h increased the basal $[Ca^{2+}]_i$ by approx. 45% in both cell types. At 50 μM, P24 induced an immediate calcium increase, which could be completely prevented by extracellular EGTA. In conclusion, we show that, in addition to the established calcium modulators cADPR and NAADP, ADP-ribosyl cyclases from mammals and invertebrates produce a family of calcium modulators with distinctive functional effects: Ap2A acts synergistically with cADPR in increasing basal $[Ca^{2+}]_i$; P18 stimulates Ca^{2+} uptake by thapsigargin-sensitive stores, thereby decreasing basal $[Ca^{2+}]_i$ i; P24 induces extracellular calcium influx through as yet unidentified channels. These dinucleotides open new perspectives on the physiology of the ADP-ribosyl cyclase/cADPR system (P24 was indeed detected together with cADPR in acid extracts of $CD38^{pos}$, but not of $CD38^{neg}$ 3T3) and may represent new pharmacological tools affecting calcium-controlled cell functions.

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Keywords: ADP-ribosyl cyclase, cADPR, calcium homeostasis; adenylic dinucleotides

MODELLING CALCIUM WAVES INITIATED WITH LOCAL AND GLOBAL PERTURBATION

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In most cell types, intracellular calcium elevation is due to agonist-stimulated inositol 1,4,5-trisphosphate (IP_3) production that allows the formation of spatiotemporal Ca^{2+} patterns, such as Ca^{2+} waves. The underlying mechanism of the propagation is not fully understood. In order to explain the phenomenon, predictions of several realistic model calculations and corresponding experimental observations were compared. Nevertheless, the method of wave initiation was different in these studies. In numerical simulations waves are initiated by local perturbation of a variable in a homogeneous medium, whereas, in experiments, in most cases, they are emerged after global perturbation, i.e. by inhomogeneous changes in the cytoplasm parameter values.

In our study, we used numerical simulations applying the Othmer-Tang model extended with a diffusional term to investigate the effects of the concentration of the second messenger IP_3 on intracellular calcium waves initiated by local and global perturbations.

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Keywords: Ca^{2+} waves, IP_3, local & global perturbation

BENZO[A]PYRENE MOBILIZES CALCIUM IN THE ASTROCYTOMA CELL LINE U373 MG

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Polycyclic aromatic hydrocarbons (PAHs), such as benzo[a]pyrene (BaP), are characterized by two or more fused carbon rings and are formed by a process of thermal decomposition of organic molecules. In general, the carcinogenicity of various PAHs correlates with their ability to suppress immune responses in animals and human lymphocytes. PAHs alter Ca^{2+} homeostasis in lymphocytes and disrupt Ca^{2+}-dependent pathways of cell activation. BaP also induces apoptosis in the human astrocytoma cell line U373 MG. This investigation was undertaken to identify the initial steps of the Ca^{2+} signaling pathway activated by BaP. The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was monitored using the visible light–excitable, high affinity Ca^{2+} indicator fluo-3.

BaP induced a fast increase in $[Ca^{2+}]_i$ followed by a slow decay to basal levels. A second application of BaP had no effect. Similar results were obtained in the presence and absence of extracellular Ca^{2+}; therefore BaP stimulation elicits an intracellular response not involving Ca^{2+} influx through plasma membrane channels. In U373 MG cells, the Ca^{2+} response after BaP was not abolished by U73122, a phospholipase C inhibitor blocking the $InsP_3$-mediated Substance P stimulation. Moreover, BaP pretreatment did not decrease the Ca^{2+} response evoked by Substance P, ryanodine or thapsigargin. All together, these results demonstrate that the $[Ca^{2+}]_i$ increase observed after BaP challenge is not originated by opening of $InsP_3$- or ryanodine-receptors or by aspecific emptying of the endoplasmic reticulum. Antimycin A blocks the respiratory chain and depolarizes mitochondria. After a 4 min antimycin A incubation, the Ca^{2+} signal induced by BaP was not observed, while further addition of thapsigargin had the expected effect. We conclude that BaP immediately mobilizes calcium from mitochondria and does not affect the other agencies regulating $[Ca^{2+}]_i$ in U373 MG cells.

Keywords: Apoptosis; Benzo(a)pyrene; Calcium; Mitochondria; Polycyclic aromatic hydrocarbon

REGULATION OF INSP^3 RECEPTOR ACTIVITY BY NEURONAL CA^{2+} BINDING PROTEINS

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Inositol 1,4,5-trisphosphate receptors ($InsP_3R$) were recently demonstrated to be activated independently of $InsP_3$ by a family of calmodulin-like neuronal Ca^{2+}-binding proteins (CaBP). We investigated the interaction of both naturally occurring long and short CaBP1 isoforms with $InsP_3Rs$, and their functional effects on $InsP_3R$-evoked Ca^{2+} signals. Using several experimental paradigms, involving transient expression in COS cells, acute injection of recombinant protein into *Xenopus* oocytes and $^{45}Ca^{2+}$ flux from permeabilised COS cells we demonstrated a decrease in the sensitivity of $InsP_3$-induced Ca^{2+} release (IICR). In addition, we found a Ca^{2+}-independent interaction between CaBP1 and the NH_2-terminal 159 amino acids of the type 1 $InsP_3R$. This interaction resulted in decreased $InsP_3$ binding to the receptor reminiscent of that observed for CaM. Unlike CaM however, CaBPs do not inhibit ryanodine receptors, have a higher affinity for $InsP_3Rs$ and more potently inhibited IICR. We also show that phosphorylation of CaBP1 at a casein kinase 2 consensus site regulates its inhibition of IICR. Our data suggests that CaBPs are endogenous regulators of $InsP_3Rs$ tuning the sensitivity of cells to $InsP_3$.

CALCIUM ENTRY THROUGH NICOTINIC ACETYLCHOLINE RECEPTORS IN DORSAL ROOT GANGLIA NEURONS

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Despite a large body of studies concerning the physiological role of Ca^{2+} entry through neuronal nicotinic acetylcholine receptors (nAChRs), very little is known about the Ca^{2+} permeability of these receptors in native systems [A]. In particular, we aimed at investigating the functional role of nAChR-mediated Ca^{2+} entry in dorsal root ganglia (DRG) neurons. These sensory neurons express a wide panel of nAChR subunit mRNAs, including α9 and α10 [B], and several well characterized nicotinic DRG current subtypes [C]. Furthermore, the increase of the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) has been shown to modulate the nociceptive DRG function, enhancing the desensitization of the capsaicin-induced response [D].

The fractional Ca^{2+} current (P_f, i.e. the percentage of current carried by Ca^{2+} ions) flowing through heteromeric nAChR expressed by rat DRG neurons, measured by fluorescence Ca^{2+} imaging used in combination with the patch-clamp technique, was 2.1±0.6 %, similar to the value previously measured, at the same membrane potential, for native heteromeric nAChRs expressed by chromaffin cells (2.5 %; [E]). The activation of these receptor-channels, by ACH or nicotine, in the presence of external Ca^{2+}, caused the elevation of $[Ca^{2+}]_i$ and the increase of the desensitization rate of the capsaicin-evoked current, along with a reduction of its amplitude. Furthermore, a long lasting incubation of DRG neurons with nicotine 200 nM, a concentration similar to that found in smoker's extracellular fluids, provoked a significant reduction of the mean amplitude of $[Ca^{2+}]_i$ transients induced by brief pulses of capsaicin.

Our results suggest that part of the analgesic properties of nAChR agonists might be due to a direct action on sensory neurons.

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Keywords: nicotinic acetylcholine receptors; dorsal root ganglia; fractional calcium current; capsaicin; pain.

CAPRI IS A NOVEL SENSOR OF Ca^{2+} ENTRY REVEALING AN ANALOGUE MODE OF Ca^{2+}-DEPENDENT RAS DEACTIVATION

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Oncogenic Ras GTPases are found in approximately 30% of human tumours and are known to be necessary and sufficient for tumour induction and maintenance in cancer models e.g. lung adenocarcinoma. These mutant proteins have defective GTPase activity, normally controlled by the binding of GTPase-activating proteins (GAPs), to which oncogenic Ras is insensitive. The canonical view of Ras signalling focuses on receptor/non-receptor tyrosine kinase signalling cascades where a Ras GEF (SOS) is recruited via adapters to the receptor in order to activate Ras at the plasma membrane. Ras is deactivated by the recruitment of p120 Ras GAP via SH2 domains to phosphotyrosine residues on the activated receptor. The delineation of this pathway a decade ago was a major advance in signal transduction research. More recently, multiple families of Ras GEFs (GRF and GRP/CalDAG-GEF) and Ras GAPs (SynGAP and GAP1) have been identified and several of these proteins appear to be regulated dynamically by diacylglycerol (DAG) and/or Ca^{2+} - each a 'product' of phospholipase C (PLC) signalling [1, 2]. Concurrently a novel class of PLC, PLCε, was discovered which operates as a Ras effector in some contexts. Thus, a battery of potential Ras signalling modulators converges with PLC-dependent second messenger pathways.

Recently, Ca^{2+} has been directly implicated in the control of Ras cycling with the discovery of twin Ca^{2+} triggered Ras GAPs: RASAL (Ras GTPase-activating-like) and CAPRI (Ca^{2+}-promoted Ras inactivator). I will present work from our lab that demonstrates their intrinsic Ras GAP function using cellular reporter assays of the Ras activation state, combined with spatio-temporal analysis of Ca^{2+}-triggered CAPRI/RASAL translocation. CAPRI and RASAL are dynamic C2 domain-dependent Ca^{2+} sensors, like conventional protein kinase C (PKC) and cytosolic phospholipase A_2. We have discovered that these GAPs sense Ca^{2+} signals very differently. RASAL senses the frequency of repetitive Ca^{2+} spikes by undergoing synchronous oscillatory association with the plasma membrane of several different *in vitro* cell lines stimulated by physiological agonists [3]. In contrast, once CAPRI reaches the membrane during the first peak of Ca^{2+} mobilisation it is rendered refractory to oscillatory changes in intracellular Ca^{2+}. In part this is due to a preferential sensing of Ca^{2+} entry over release, consistent with the sensitivity of CAPRI to store-operated Ca^{2+} entry. Thus CAPRI and RASAL can differentially sense the same Ca^{2+} signal to enable both frequency-dependent and analogue modes of Ras regulation. In addition, the behaviour of CAPRI suggests that Ca^{2+} entry can mediate Ras deactivation which has important implications for the role of extracellular Ca^{2+} in Ras-dependent cellular proliferation and protection from apoptosis.

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Keywords : Calcium, Ras, GAP, C2 domain, CAPRI, RASAL

ALTERED LPS SIGNALING IN S100A9 -/- MICE

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S100A8/S100A9 expressing phagocytes are the first infiltrating cells in acute inflammatory lesions. Both proteins belong to the multigenic family of S100 proteins comprising 21 members to date. Common to all S100 proteins are the homodimerisation and the capacity to bind bivalent ions. Typically for S100A8/S100A9 is the tissue specific expression in monocytes and granulocytes, the ability to form heterodimers, and the immense abundance in granulocytes. There is increasing evidence that both S100 proteins play regulatory roles within phagocytes. In addition, both molecules are released into the extracellular space to exert effects on target cells. While mice deficient in S100A8 (Passey et al., 1999) die during embryogenesis, S100A9 -/- mice (Manitz et al., 2003) show no obvious phenotype.

To get more insight into the function of S100A9 we compared the gene expression pattern of bone marrow cells of wild-type mice and S100A9 -/- mice using Affymetrix GeneChip expression arrays containing ~11,800 probes. The most prominent changes were found after bacterial endotoxin/lipopolysaccharide (LPS) activation. Interestingly, the basal expression of 32 out of 37 genes which were up- or downregulated in S100A9 -/- cells compared to wild-type cells showed a parallel regulation in wild-type cells after LPS activation. This indicates, that S100A9 -/- cells are in a ´pre-activated´ state.

To further characterize the S100A9 -/- cells we investigated LPS signaling in vivo. In a septic shock mouse model (low dose LPS model) S100A9 -/- mice show prolonged survival rates concomitant with lower TNFα expression level. LPS activates the p38 kinase signaling cascade responsible for induction of TNFα and other cytokines. Interestingly, knocking out of MK2, the next downstream kinase of the p38 pathway in mice results in a similar phenotype as observed in S100A9 -/- mice. However, in addition to parallels also differences are obvious to the phenotype of MK2 -/- mice, which are currently under investigation.

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Keywords: S100A9, S100A8, neutrophils, knockout mice, LPS, p38 pathway

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

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S100 proteins are small dimeric proteins which comprise a large subfamily within the EF-hand superfamily of Ca^{2+} binding proteins. They are thought to participate in mediating intracellular Ca^{2+} signals by binding to and thereby regulating target proteins in a Ca^{2+} dependent manner. Affinity chromatographory studies with the biologically active S100P dimer identified ezrin, a membrane/ F-actin crosslinking protein, as a dimer-specific S100P ligand. The formation of the S100P-ezrin complex is Ca^{2+} dependent and occurs via the N-terminal domain of ezrin (N-ERMAD). This domain is accessible for interaction in dormant ezrin, in which the membrane and F-actin-binding-sites are masked through interactions between the N-terminal and C-terminal domain. Interestingly F-actin cosedimentation assays with purified ezrin and WT S100P showed that the binding of S100P unmasks the F-actin binding site in the C-terminal domain of ezrin thereby activating the ezrin molecule. This identifies S100P as a novel activator of ezrin and indicates that an activation of ezrin`s membrane/ cytoskeleton crosslinking funktion can occur directly in response to Ca^{2+} transients.

In order to identify specific amino acid residues involved in S100P-ezrin complex formation we also generated a series of C-terminal truncated N-ERMAD and S100P derivates and analyzed their interactions with WT S100P and WT N-ERMAD respectively. Results dilineating the sites of complex formation will be discussed.

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

CALCIUM SIGNALLING DEFECT IN S100A9 KNOCKOUT NEUTROPHILS

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S100A9 and its heterodimeric partner, S100A8, are cytosolic calcium-binding proteins highly expressed in neutrophils and monocytes. To understand the function of S100A9, we performed targeted disruption of the S100A9 gene in mice. S100A9$^{-/-}$ mice showed no gross phenotype and were fertile. S100A8 mRNA but not protein was present in the myeloid cells of these mice, making them effectively S100A8/9$^{-/-}$ cells. A compensatory increase in other proteins was not detected in the S100A8/9$^{-/-}$ cells.

Ca^{2+} signalling in the S100A8/9$^{-/-}$ neutrophils was examined by flow cytometry using Indo-1. As there was no difference in thapsigargin induced calcium flux between wildtype and S100A8/9$^{-/-}$ cells, the calcium stores and store operated calcium entry pathways must be broadly normal. The response to ionomycin was also identical in wildtype and S100A8/9$^{-/-}$ cells, indicating that the S100A8/9 complex is not a major cellular calcium buffer. When Ca^{2+} response to the chemokine MIP-2 was studied a decreased Ca^{2+} response to sub-maximal concentrations of chemokine was observed in the S100A8/9$^{-/-}$ cells. Further studies with a range of other chemokines including KC and C5a revealed a similar defect in Ca^{2+} flux. Interestingly when the chemotactic peptide FMLP was titrated, the S100A8/9$^{-/-}$ cells showed no defect, even at low levels of stimulus. Examination of Ca^{2+} flux in the absence of extracellular calcium revealed the defect to be in intracellular calcium release. Inhibitor studies appear to localise the defect to the IP_3 mediated calcium release pathway. Studies to characterise this defect and its functional significance are ongoing.

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Hobbs, J, et al. Myeloid Cell Function in MRP-14 (S100A9) Null Mice. Mol Cell Biol. Apr;23(7):2564-76, 2003

Keywords : S100A9; S100A8; Neutrophil; Knockout; Defect;

INSIGHTS INTO Zn^{2+}-BINDING SITES IN S100 PROTEINS FROM THE CRYSTAL STRUCTURE OF Ca^{2+}/ZN^{2+} S100B AND SPECTROSCOPIC INVESTIGATIONS OF Zn^{2+}-S100A2

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The S100 protein family is the largest subgroup within the Ca^{2+}-binding EF-hand superfamily [1] In addition to Ca^{2+}, several members of the S100 protein family bind Zn^{2+} and Cu^{2+} with high affinity [2]. One of our aims is to elucidate the structures of the Zn^{2+}/Cu^{2+} binding sites in S100 proteins and to understand the function of Zn^{2+}/Cu^{2+}-binding. We succeeded in crystallization of Ca^{2+}/Zn^{2+} loaded S100B and solved the structure at 1.85 Angstrom resolution. The observed Zn^{2+}-binding site in the crystal structure is located at the interface of the S100B homodimer and is formed by histidine and carboxylate ligands originating from both S100B subunits. Overall the binding site resembles the Cu^{2+} binding site observed in the structure of Ca^{2+}/Cu^{2+}-S100A12. Spectroscopic investigations of Cu^{2+}-S100B suggest that Cu^{2+} binds to the same site as Zn^{2+}.

High-affinity Zn^{2+}-binding is reported for S100A2, too. However, the residues involved in Zn^{2+}-binding in S100B are not conserved in S100A2. Therefore we explored the Zn^{2+}-binding sites of S100A2 by a combination of homology modelling, site-directed mutagenesis and UV-Vis, CD, MCD and EPR spectroscopy. The results revealed two different Zn^{2+}-binding sites in S100A2, which are formed by cystein and histidine residues. The first site is located at the interface of the homodimeric S100A2 formed by His17, Cys21 and the protein backbone. Interestingly, Cys21 is located in the N-terminal EF-hand influencing the Ca^{2+}-binding properties of S100A2. In the second Zn^{2+}-binding site Cys2 serves as a ligand, whereby Zn^{2+} bridges twoS100A2 homodimers and triggers the formation of a tetrameric species. Thus we propose that Zn^{2+} acts as a regulatory ion in S100A2 dependent signalling pathways.

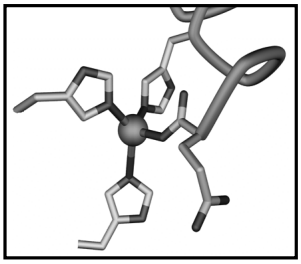

Figure1. Zn^{2+}-binding site in S100B

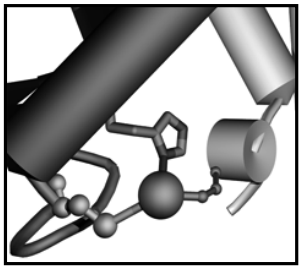

Figure 2. Zn^{2+}-binding site in S100A2

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Keywords : S100B, S100A2, Zinc, Copper, X-ray, structure

EXTRACELLULAR S100A4(MTS1) STIMULATES INVASIVE GROWTH OF MOUSE ENDOTHELIAL CELLS AND MODULATES MMP-13 MATRIX METALLOPROTEINASE ACTIVITY

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S100A4(mts1) protein expression has been strongly associated with metastatic tumor progression. It has been suggested as a prognostic marker for a number of human cancers. It is proposed that extracellular S100A4 accelerates cancer progression by stimulating motility of endothelial cells, thereby inducing angiogenesis. Here we show that in 3D culture mouse endothelial cells (SVEC 4-10) respond to the treatment with recombinant S100A4 by stimulation of an invasive growth of capillary-like structures. The outgrowth is not dependent on stimulation of cell proliferation, but rather correlates with modulation of transcription of genes involved in proteolytic degradation of extracellular matrix. Treatment of SVEC 4-10 with the S100A4 protein lead to activation of transcription of collagenase 3 (MMP-13) mRNA followed by subsequent release of the protein from the cells. β–casein zymography demonstrates enhancement of proteolytic activity associated with collagenase 3. This observation indicates that extracellular S100A4 stimulates production of extracellular matrix degrading enzymes from the endothelial cells thereby stimulating remodeling of extracellular matrix. This could explain the angiogenic and metastasis –stimulating activity of S100A4(mts1).

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Key words: metastasis-promoting S100A4, extracellular activity, MMP-13 induction.

CALCIUM BINDING TO FIBRILLIN-1 TB-cbEGF DOMAIN PAIRS: NEW INSIGHTS INTO MICROFIBRIL ORGANISATION

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Calcium-binding epidermal growth factor-like (cbEGF) and transforming growth factor _-binding protein-like (TB) domains are dominant features of the structure of the glycoprotein fibrillin-1, the major structural component of the 10 - 12 nm extracellular matrix microfibrils. TB-cbEGF interdomain interactions have significant implications for the organisation of fibrillin-1 molecules within in the microfibril. We measured the calcium dissociation constants of all six fibrillin-1 TB-cbEGF pairs and studied the interdomain contacts that influence calcium binding by site directed mutagenesis. The calcium dissociation constants (K_d) of fibrillin-1 TB-cbEGF domain pairs varied widely, from 10 nM to 1.6 mM. Hydrophobic contacts at the interdomain interface contributed significantly to the stability of calcium binding and high resolution studies by 2D NMR showed that TB-cbEGF interface formation is strongly calcium-dependent. These results highlight the role of calcium in fibrillin interdomain interactions and provide new constraints for the organisation of fibrillin within the 10 - 12 nm microfibrils.

Keywords : extracellular matrix, fibrillin, cbEGF, calcium, NMR

CALMODULIN BINDING PROTEINS AND NUCLEAR PORES SHAPE THE KINETICS OF CALCIUM INDUCED TRANSLOCATION OF CALMODULIN

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It has been demonstrated in pancreatic acinar cells (Craske *et al.* 1999) that calmodulin (CaM) undergoes intracellular redistribution after application of the calcium-releasing agonists, acetylcholine (ACh) and cholecystokinin (CCK). A large cytosolic calcium transient generated by a supra-maximal dose of ACh or CCK caused a rapid rise of [CaM] in apical region and a rapid drop in the basal region while the [CaM] in the nucleus rose more slowly, and lasted long after the termination of the calcium transient. Global cytosolic calcium spikes generated by 50 pM CCK caused [CaM] spikes in the apical region that resembled cytosolic calcium spikes, and [CaM] in the basal region showed the mirror image of the apical [CaM] spikes with smaller magnitude. On the other hand, [CaM] in the nucleus showed a delayed, slower and steadier increase that stabilized in about 100s.

In order to understand these behaviours of [CaM] during the agonist stimulations, we developed computational models using FEMLAB, an interactive environment for modelling mathematical problems based on a system of coupled partial differential equations. The model had two-dimensional geometry with three spatial domains which represented the nucleus, apical and basal cytosolic regions. We assumed that calcium-free calmodulin (apoCaM) and calcium-bound calmodulin (CaCaM) could diffuse freely in the cytosol or in the nucleus, or bind to non-diffusible binding partners (apoCaM-binding proteins and CaCaM-binding proteins, respectively). It was also assumed that the diffusion between the cytosol and the nucleus was limited by nuclear pores. Parameters in the model were estimated using the time series data of [CaM] during 1nM CCK stimulations and global optimisation algorithms. Our computational simulations showed that (1) heterogeneous distribution of calmodulin binding proteins can be modelled and quantified as the difference of calmodulin binding capacities (the ratio between CaM bound to its targets and freely diffusible CaM), (2) the slow kinetics of nuclear CaM can be due to the limited diffusion across the nuclear pore as well the slow release of CaM from CaCaM binding proteins, (3) estimated first order rate constant of the CaM diffusion across the nuclear envelop in the acinar cell was similar to the value in peameabilized smooth muscle cell line (first order rate constant = $0.013s^{-1}$, Liao *et al*, 1999), indicating the similality of nuclear pore permeability between two cell types. Our computational models can be used to extract quantitative information from spatial dynamics of signalling proteins.

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Keywords: Calmodulin, computational model, pancreatic acinar cells, nucleo-cytoplasmic transport.

SKELETAL MUSCLE SARCOLEMMA IS THE TARGET OF CHOICE FOR CHOLESTEROL DEPLETING DRUGS

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Cholesterol-lowering therapy using statins (HMG CoA reductase inhibitors) is exceedingly common in an aging population suffering from cardiovascular diseases. However, muscle pain and weakness are frequent adverse events. The plasma membrane of skeletal muscle is divided into the lateral load-bearing region (the sarcolemma) and, emanating from it, multiple transverse membrane infoldings (the T-tubular system) which surround each myofibril and mediate excitation-contraction coupling.

Extraction of membrane cholesterol with methyl-beta cyclodextrin suggests that T-tubular structures are more vulnerable to cholesterol depletion than the lateral sarcolemma. Fine structural analysis using ultrathin cryosectioning and labelling of distinct membrane regions with specific antibodies against cholesterol-associated proteins, reveals that sarcolemma and T-tubular membranes are distinct in contents and nature of their cholesterol-binding proteins. While sarcolemmal cholesterol is largely stabilized by caveolin, the T-tubular membranes lack caveolin but contain annexins 2 and 6, known to only temporarily associate with membrane cholesterol depending on $[Ca^{2+}]_i$.

The inhibition of HMG CoA reductase in mice and growing rats led to the development of myopathic changes, which closely resemble the structural damage inflicted by cyclodextrin treatment in vitro, and injuries reported in human patients undergoing cholesterol-lowering statin therapy.

Our experiments support the hypothesis that the unique lipid-protein segregation is responsible for the vulnerability of skeletal muscle sarcolemma and its structural damage following cholesterol depletion.

Keywords: annexins – sarcolemma – muscle – statins – HMG CoA reductase

TARGETED INHIBITION OF Ca^{2+}/CALMODULIN-DEPENDENT KINASE II IN CARDIAC MYOCYTES OF TRANSGENIC MICE

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Ca^{2+}/calmodulin-dependent kinase II regulates in many cellular events in cardiac myocytes including Ca^{2+} cycling, contraction and transcription. We have designed and constructed transgenes that target the expression of a potent peptide inhibitor of CaM KII, AIP, to the longitudinal sarcoplasmic reticulum (LSR-AIP_4) and the nucleus (NLS-AIP_4). The LSR-AIP_4 transgenic mice demonstrated a 60-70% decrease in phospholamban phosphorylation at threonine-17 under basal and β-adrenergic stimulated conditions without changing phospholamban phosphorylation at serine-16. Phosphorylation of RyR2 in the junctional SR was unchanged. Ca^{2+} uptake assays with isolated SR vesicles showed that there was a 30% decrease in V_{max}. The *in vivo* measurement of cardiac function showed no change in positive and negative dP/dt. There was, however, an increase in the time constant of relaxation. Younger transgenic mice (7 months) display no signs of cardiac disease unless challenged by cardiovascular work, such as gestation and parturition or increased afterload (aortic banding). These mice develop progressive dilated heart failure. The nuclear-targeted NLS-AIP_4 transgenic mice demonstrate a reduction in the phosphorylation of CREB (serine-133) in response to β–adrenergic stimulation. These animals display no overt phenotype; they are being evaluated for their cardiovascular response to cardiac hypertrophic challenges. These studies collectively demonstrate that the subcellular localization of CaM KII allows for independent regulation of specific myocyte functions.

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Keywords: Cardiac, Ca^{2+}/calmodulin-dependent kinase II (CaM KII), AIP_4 (CaM KII inhibitor), subcellular targeting sequences, transgenic mice

Ca^{2+}-INDEPENDENT AND Ca^{2+}-DEPENDENT MECHANISMS OF CALMODULIN NUCLEAR TRANSLOCATION

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Calmodulin translocation mechanisms[1-4] are investigated in HeLa, rat glial and neuronal cells by confocal microscopy. Cells are electroporated with fluorescently labelled calmodulins and dextrans. Three states of permeability of the nuclear pores are identified: 1) minimally open, 2) normally open and 3) enlarged. State 1 is observed in low intracellular [Ca^{2+}]; state 2 occurs in Ca^{2+} stimulated cells and state 3 is induced by Ca^{2+}/calmodulin. Coincidentally, in state 1 the intracellular Ca^{2+} stores are depleted and in state 2 they are slowly refilling, it is however not evident whether the difference in pore size between states 1 and 2 is the direct effect of Ca^{2+} or attributable to the state of the stores[5]. Permeability in state 3 is independent of the Ca^{2+} content of the stores. Diffusion of a 19.5 kDa dextran into the nucleus in states 1 and 2 is slow with $t_{1/2}$ >> 1 h and $t_{1/2}$ = 30 min, respectively. Calmodulin entry into the nucleus is blocked by wheat germ agglutinin in both states 1 and 2. Calmodulin translocation into the nucleus upon intracellular Ca^{2+} elevation occurs with a $t_{1/2}$ of 120 s. These data show, in all three cell types examined, that free diffusion of calmodulin and of Ca^{2+}/calmodulin is too slow to significantly contribute to the nuclear entry of calmodulin upon Ca^{2+} stimulation or to the re-equilibration of calmodulin between the cellular compartments following a Ca^{2+} transient. Calmodulin nuclear translocation in response to stimulus thus occurs by facilitated transport both in the absence and in the presence of Ca^{2+}. Ca^{2+}-dependent calmodulin entry into the nucleus is inhibited by the membrane permeant calmodulin binding mTrp peptide. This transport mechanism is thus specific for Ca^{2+}/calmodulin.

Typically 7 min after Ca^{2+} stimulation, a further influx of calmodulin into nucleus occurs and at the same time, dextrans up to 70 kDa in mass will also enter the nucleus. This process proceeds with a $t_{1/2}$ of 70 s and is inhibited by a Ca^{2+}/calmodulin binding peptide. These data show that Ca^{2+}/calmodulin induces a significant enlargement of the nuclear pore size.

Our data demonstrate both Ca^{2+}-independent and a Ca^{2+}-dependent facilitated calmodulin translocation mechanisms into the nucleus and show a role for Ca^{2+}/calmodulin in the regulation of the nuclear pore size.

This work is supported by the MRC and the Wellcome Trust.

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Keywords: nuclear pore, calmodulin, diffusion, facilitated transport

IQGAP1 RECRUITS CALMODULIN TO THE CORTEX WHERE IT MODULATES CYTOSKELETAL REORGANIZATION

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IQGAP1, an integral component of cytoskeletal function, binds to a diverse array of signalling and structural molecules, including actin, the microtubule binding protein CLIP-170 and calmodulin (CaM) (1). CaM is diffusely distributed throughout cells with accumulation in two locations; the spindle poles and the cell cortex (2). In rat basophilic leukaemia (RBL-2H3) cells, CaM is found concentrated at both locations (3) where in resting primary rat peritoneal mast cells, localizes at the cell cortex where it regulates cortical actin disassembly which precede late steps of exocytosis (4).

Here we report that IQGAP1 is found at the cell cortex of permeabilised human mast cells (HMC-1) and that calcium enhances this localization. IQGAP1 was specifically knocked down by small interfering RNA (siRNA) in order to evaluate its role in spreading and secretion. Compared to control RBL cells, knock down of IQGAP1 enhanced both spreading and secretory responses following stimulation by either antigen or by phorbol plus calcium ionophore. Moreover, in stably expressing EGFP-tagged CaM RBL cells down-regulation of IQGAP1 eliminated the cortical concentration of CaM but did not affect its spindle pole localization. Our results suggest that IQGAP1 is responsible for targeting CaM to the actin cortex where it can influence exocytosis.

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Keywords : calmodulin, actin, mast cells, siRNA, IQGAP

CONCENTRATION OF CALBINDIN-D28k IN HIPPOCAMPAL NEURONS

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Calbindin-D_{28k} is an intraneuronal Ca^{2+} binding protein that is expressed in many types of neurons throughout the CNS. Especially granule cells of the hippocampus show a very strong immunoreactivity for Calbindin-D_{28k}.[1] Ca^{2+} binding proteins are capable of modulating different intracellular Ca^{2+} signals: If present at concentrations of several hundred micromolar they can influence elevations of the cytosolic Ca^{2+} concentration (so called "bulk calcium") as well as fast submembraneous Ca^{2+} profiles in the immediate vicinity of Ca^{2+} channels (so called "calcium microdomains"). Despite the knowledge of *in vitro* Ca^{2+} binding properties[2], the role of Calbindin-D_{28k} *in vivo* is poorly understood because its physiological concentration is not known. The aim of the presented study was to determine the intraneuronal Calbindin-D_{28k} concentration with the use of whole cell patch clamp recordings and post recording immunohistochemistry of hippocampal granule cells. First, we investigated the intracellular mobility of Calbindin-D_{28k}. We interrupted whole cell recordings at different time points and stained slices for Calbindin-D_{28k}. With increasing duration of the whole cell recording we observed a decrease of the Calbindin-D_{28k} immunoreactivity of the measured cell, that could be well fitted by an exponential function yielding a time constant of 10 min. The time constant suggests that Calbindin-D_{28k} freely diffuses between the cytoplasm and the patch pipette during a whole cell recording[3]. Next, we made use of the fact that the intracellular Calbindin-D_{28k} concentration can be controlled by the pipette solution. We proceeded similar but added different concentrations of purified Calbindin-D_{28k} to the pipette solutions and held the cells 30 min in whole cell mode. Our experiments show that ~32 µM of purified Calbindin-D_{28k} are sufficient to let the measured cell appear as bright as the surrounding neurons in the same visual field. Based on the variability of their immunoreactivity, we estimate that individual granule cells contain 20 - 40 µM calbindin-D_{28k}. In conclusion our results indicate that 1. Calbindin-D_{28k} is a mobile intracellular Ca^{2+} binding protein. 2. Granule cells contain up to 40 µM Calbindin-D_{28k} equivalent to 160 µM Ca^{2+} binding sites. 3. The endogenous Calbindin-D_{28k} concentration constitutes a Ca^{2+} binding ratio, K_S, of > 200. This high cytoplasmic Ca^{2+} buffer capacity of Calbindin-D_{28k} suggests a strong influence on cellular Ca^{2+} transients.

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Keywords : Calbindin-D_{28k}, bulk calcium, calcium microdomains, hippocampal granule cell

FAST CALCIUM SIGNALING TO THE NUCLEUS: ACTIVATION OF GENE TRANSCRIPTION WITHIN SECONDS AFTER ELECTRICAL ACTIVATION OF HIPPOCAMPAL NEURONS

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Electrical activity-induced calcium signals can activate gene transcription in neuron. Synapse-to-nucleus communication takes place either via calcium-regulated protein kinase cascades (such as the ERK-MAP kinase pathway triggered in the vicinity of the calcium entry channel) or via calcium itself that can invades the cell nucleus (1). Signaling to the nucleus via protein kinase cascades is expected to be slower than signal propagation by calcium that is detected in the nucleus within a second after electrical activation. The speed of the activation of genomic responses may therefore provide information on the mode of calcium signal propagation used. Using the cellular compartment analysis of temporal activity by fluorescence in situ hybridization (catfish) (2) the temporal profile of transcription of the immediate early gene *Zif268* (also known as *Egr-1*, *Krox-24*, *NGFI-A*) was assessed in rat hippocampal neurons after depolarization with 50 mM extracellular KCl. Individual *Zif268* intranuclear foci were detected after 30 sec of KCl treatment. These intranuclear foci (INF) are the genomic sites of *Zif268* transcription. Particularly at later time points, *Zif268* RNA can also be seen in a characteristic cytoplasmic localization pattern outside of the nucleus. Pharmacological experiments were done to examine the contribution of the ERK-MAP kinase pathway and the p38 MAP kinase pathway to KCl-induced *Zif268* transcription.

Supported by the Alexander von Humboldt Foundation

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Keywords: Calcium signaling, gene transcription, *Zif268*, in situ hybridization

SPONTANEOUS SYNAPTIC ACTIVITY IN HIPPOCAMPAL NEURONS REGULATES THE EXPRESSION OF THE $INSP_3R$ AND CONFERS SENSITIVITY TO EXCITOXICITY

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In this study, we investigated the effects of spontaneous calcium signals, resulting from synaptogenesis, on the maturation of metabotropic responses and the effect these oscillations have on sensitivity to glutamate induced excitotoxicity.

1-2 day old rat pup hippocampal neurons cultured at 'high-density' (35,000 cells/cm^2) began to exhibit spontaneous, synchronous oscillations in intracellular calcium concentration from day 11 in culture. This correlated with the development of intracellular calcium responses to the metabotropic receptor agonists: carbachol and (RS)-3,5-dihydoxyphenylglycine. In contrast, neurons cultured at 'low-density' (5,000 cells/cm^2) demonstrated an absence of spontaneous activity and with that, a complete lack of response to carbachol and DHPG during any stage of development.

Direct stimulation of the inositol 1,4,5 trisphosphate receptor using 30 μM inositol 1,4,5 trisphosphate ester elicited a large calcium signal only in mature 'high-density' neurons. In contrast, low-density neurons exhibited significant protection from glutamate-induced de-regulation of the intracellular calcium concentration, as did immature high-density neurons. Mature high-density neurons showed a faster rate of calcium de-regulation and with that a significant decrease in the delay before propidium iodide influx into neurons, indicative of cell death.

We conclude that the oscillations in intracellular calcium concentration lead to the maturation of the metabotropic calcium signalling 'toolkit', but simultaneously confer sensitivity to excitotoxic insults.

Supported by: BBSRC and Merck Sharp and Dohme

Keywords : neuron, synaptogenesis, calcium, excitotoxicity.

THE LOCALISATION AND BEHAVIOUR OF NEURONAL CALCIUM SENSOR PROTEINS IN HIPPOCAMPAL NEURONS

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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 (Burgoyne *et al*, 2004). The NCS proteins possess four EF-hand domains, with the most N-terminal of the hands unable to bind Ca^{2+}. In certain NCS proteins, this first EF-hand is involved in the Ca^{2+}/myristoyl switch, in which binding of Ca^{2+} 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 laboratory has demonstrated that at resting cytosolic [Ca^{2+}], NCS-1, K^+ channel interacting protein–1 (KChIP-1), and a related Ca^{2+} binding protein; Ca^{2+} binding protein–1 (CaBP-1) are constitutively associated with their target membranes when expressed in HeLa cells (O'Callaghan *et al*, 2002 ;O'Callaghan *et al*, 2003b; Haynes *et al*, 2004). The above proteins, therefore, do not demonstrate a functional Ca^{2+}/myristoyl switch. In contrast, this is not the case for hippocalcin (O'Callaghan *et al*, 2003a). Whilst hippocalcin is present in the cytosol at resting [Ca^{2+}], stimuli resulting in an increase of [Ca^{2+}] result in a concomitant translocation of hippocalcin to the trans Golgi network (TGN), and the plasma membrane (PM). We show here 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. We 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.

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Key words: Calcium binding proteins, Neuronal calcium sensors, Hippocalcin, NCS-1, Neurons

THE NEURONAL CALCIUM SENSOR PROTEIN KChIP1 AND ITS ROLE IN Kv4.2 POTASSIUM CHANNEL TRAFFIC

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The Neuronal Calcium sensor (NCS) proteins are EF-hand containing Ca^{2+}-binding proteins that are predominantly expressed in neurons where they have been implicated in the regulation of a range of cellular processes.[1,2] The KChIPs (K^+ channel interacting proteins) belong to the NCS protein family and act as auxiliary subunits of Kv4 potassium channels. As well as regulating the gating properties of the channel they increase the Kv4 current density in a Ca^{2+}-dependent manner.[3] This suggests that KChIPs have the effect of stimulating the traffic of Kv4 channels to the plasma membrane, although the underlying mechanism is unkown.

Here we investigated the role of KChIP1 in membrane traffic and the importance of its Ca^{2+} sensing ability. Using GFP-variant fusion proteins of KChIP1 and Kv4.2 expressed in HeLa and PC12 cells, we explored the individual targeting of the two proteins and their potential site of interaction in the secretory pathway. ECFP-Kv4.2 expressed alone reached the Golgi apparatus but required KChIP1 to traffic to the plasma membrane. The targeting of KChIP1 is determined by its minimal myistoylation sequence[4] and when expressed alone KChIP1-EYFP was targeted via its myristoyl tail to punctuate structures, which had the characteristics of post-ER transport vesicles that moved in a microtubule dependent way. Myristoylation and correct targeting of KChIP1 were required for efficient traffic of Kv4.2 to the plasma membrane. Ca^{2+}-binding to KChIP1 was also necessary for its effect on Kv4.2 plasma membrane association. The EF-hand mutant of KChIP1, which was unable to bind Ca^{2+}, stopped ECFP-Kv4.2 from reaching the Golgi apparatus, which suggests that the traffic to the Golgi was a Ca^{2+}-dependent step.

In summary, our data indicates that the site of interaction of KChIP1 and Kv4.2 is at the post-ER pre-Golgi level and at this stage Ca^{2+} sensing by KChIP1 is essential for any further traffic of the Kv4.2-KChIP1 complex through the secretory pathway and for it to reach the plasma membrane.

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CALCIUM EXTRUSION PROTEIN EXPRESSION IN THE HIPPOCAMPAL FORMATION OF CHRONIC EPILEPTIC RATS AFTER KAINATE-INDUCED STATUS EPILEPTICUS

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The plasma membrane Ca^{2+}-ATPase (PMCA) and (potassium-dependent) sodium-calcium exchange (NC(K)X) represent two main calcium extrusion mechanisms that are important for the restoration of $[Ca^{2+}]_i$ levels after electrical activity. To investigate whether the expression of these calcium extrusion proteins is altered in the course of epileptogenesis, we compared the hippocampal-parahippocampal protein expression of NCX1, 2, and 3, PMCA1-4, and NCKX2 at an early and late stage after kainate-induced status epilepticus (SE), with that in control rats using immunocytochemistry. Several alterations were found in chronic epileptic rats: (i) NCX1 was permanently downregulated in the inner molecular layer (iml) of the dentate gyrus (DG) and entorhinal cortex layer III (ECIII), related to neuronal loss in hilus and ECIII, respectively; (ii) PMCA and NCKX2 expression was transiently upregulated in the iml, and downregulated in several areas where cell loss had occurred, (iii) NCX3 expression, which in control rats is abundant in presynaptic terminals of mossy fibers (mf), was extensively and permanently decreased in stratum lucidum and hilar region. In addition, newly formed mf sprouts that project to the DG iml did not noticeably express NCX3; (iv) NCX2 and NCKX2 were (transiently) upregulated in astrocytes of epileptic rats throughout the hippocampal formation, including ECIII. Our results show regional-specific changes of calcium extrusion proteins in epileptic rats that contribute to altered calcium homeostasis in the diseased state. More importantly, some alterations in calcium extrusion protein expression are already present at an early stage of epileptogenesis and could therefore be involved in this process.

Keywords: NCX, PMCA, extrusion, epileptogenesis, immunocytochemistry, hippocampus.

INTRACELLULAR S100B REGULATES ASTROCYTIC INTERMEDIATE FILAMENT ASSEMBLY AND NEURITE OUTGROWTH IN VITRO

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Objective
In several neurodegenerative diseases the secretion of the astrocytic protein S100B is upregulated. Its role in the pathogenesis of these disorders, however, remains to be characterized. Under certain circumstances such as ischemic conditions upregulated extracellular S100B is accompanied by low intracellular S100B content. In protein binding assays it was shown that S100B inhibits intermediate filament assembly of GFAP and vimentin by inhibition of protein kinase C mediated phosphorylation. Here we show that in primary cultured astrocytes from wildtype and S100B$^{-/-}$ mice S100B regulates GFAP, vimentin and nestin intermediate filament assembly influencing neurite outgrowth and neuronal survival.

Methods and Results
Intermediate filament density was increased in S100B$^{-/-}$ cortical astrocytes in situ identified by electron microscopy. Knockout-proven S100B immunoreactivity was co-localized with the filamentous structures of GFAP in cultured astrocytes. In S100B$^{-/-}$ astrocytes GFAP and Vimentin were upregulated whereas nestin immunoreactivity was reduced compared to wildtype astrocytes. As a consequence, laminin expression was downregulated on S100B$^{-/-}$ astrocytes leading to a reduced neurite outgrowth and neuronal survival after 4 days of co-culture with S100B$^{-/-}$ astrocytes shown by b-Tubulin III immunoreactivity. This was caused by intracellular S100B since recombinant S100B protein added to the wildtype or S100B$^{-/-}$ astrocyte culture medium had no effect on intermediate filament protein expression.

Conclusion
Intracellular S100B content regulates the intermediate filament protein assembly in astrocytes. Downregulation of intracellular S100B leads to lower production of laminin by astrocytes and inhibition of neurite outgrowth. Our data indicate a pathway how S100B might be involved in the pathogenesis of neurodegenerative diseases.

Ca^{2+}-INDUCED CONFORMATIONAL CHANGES IN APOPTOSIS-LINKED GENE-2 AND IMPLICATIONS FOR ITS PROTEIN INTERACTIONS IN OCULAR MELANOMA

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Apoptosis-Linked Gene 2 *(ALG-2)* encodes a 22 kDa Ca^{2+}-binding protein of the penta- EF-hand family that is required for programmed cell death in response to various apoptotic agents. Here, we demonstrate that *ALG-2* mRNA and protein are down-regulated in human ocular melanoma cells compared to their progenitor cells, normal melanocytes. The down-regulation of ALG-2 may provide melanoma cells with a selective advantage.

ALG-2 was found to be predominantly cytosolic in both melanocytes and melanoma cells and was localized in the same manner as its putative target molecule, Alix/AIP1, independent of the intracellular [Ca^{2+}] or the activation of apoptosis. Fluorescence studies of ALG-2 demonstrated that an increase in surface hydrophobicity is primarily due to Ca^{2+} binding to EF-3, while Ca^{2+} binding to EF-1 or EF-5 had little effect on surface exposure of hydrophobic residues. However, binding of Ca^{2+} to both EF-1 and EF-3 is necessary for ALG-2 interaction with Alix/AIP1 as demonstrated using surface plasmon resonance spectroscopy (SPR). The interaction is reduced but not eliminated when binding of Ca^{2+} to EF-5 is compromised. The N-terminal non EF-hand region of human ALG-2 was found nonessential for dimer formation, calcium-induced fluorescence changes or target interaction[1].

Similar to Alix/AIP1, mutations affecting EF-1 and EF-3 prevent the interaction of ALG-2 with another potential calcium-dependent target, the N-terminal region of Annexin XI. In addition, mutations of aromatic residues lining the hydrophobic pocket identified in the crystal structure of ALG-2[2], result in decreased interaction with Annexin XI, as measured by SPR. Therefore, the hydrophobic pocket exposed as a result of calcium-induced conformational changes in ALG-2 is likely a principal site of target interaction.

The down-regulation of ALG-2 in melanoma cells has interpretable consequences for a cancer phenotype. Recent literature suggests that ALG-2 is involved in the ER (endoplasmic reticulum) stress-induced intrinsic apoptotic pathway, responding to aberrations in calcium homeostasis[3]. The down-regulation of ALG-2 may help cancer cells sever the connection between elevated levels of intracellular calcium in response to ER stress and the activation of degradative enzymes that start the apoptotic process.

Understanding the mechanism of calcium-induced conformational changes of ALG-2 and the consequent sites of interaction with downstream targets could lead to opportunities for therapeutic intervention in ocular melanoma and related diseases.

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OVEREXPRESSION OF S100B IN RAT L6 MYOBLASTS RESULTS IN INCREASED PROLIFERATION AND REDUCED DIFFERENTIATION

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Cell proliferation and differentiation are mutually exclusive processes that in general rely on the activation of entirely distinct gene programs. For example, at a certain stage during development, myoblasts, i.e. the precursors of skeletal muscle cells, cease to proliferate and start to differentiate where and when they had reached a critical density (1). In addition to the cell number, a combination of events is responsible for the switch from proliferation to differentiation, such as the exchange of extracellular signals acting in a paracrine and/or autocrine manner, the secretion of specific components of the extracellular matrix, the exposure of molecules on the cell surface that recognize extracellular signals and/or components of the extracellular matrix, and the presence in the extracellular space of factors with ability to modulate cell differentiation. Insulin and insulin-like growth factors are canonical stimulators of myoblast differentiation, while bFGF, TGF-β and TNF-α are recognized inhibitors of myogenesis. We recently reported that S100B, which is normally found in extracellular fluids in pM amounts, inhibits myogenesis in vitro by interacting with a non-RAGE receptor with K_d ~40 pM (2) and overrides the promyogenic effects of RAGE engagement by amphoterin in rat L6 myoblasts (3). As myoblasts normally express S100B and S100B expression in skeletal muscle fibers is developmentally regulated (4,5), we sought to investigate the potential role of intracellular S100B in rat L6 myoblast terminal differentiation. Experiments were carried out on L6 myoblasts stably overexpressing S100B (L6/S100B) and mock-transfected myoblasts (L6/Mock). Overexpression of S100B in L6/S100B myoblasts was documented by RT-PCR and Western blotting. L6/S100B myoblasts proliferated at a higher rate compared with L6/Mock myoblasts and, under differentiation conditions, they did not fuse into myotubes. Also, little or no expression of the muscle-specific transcription factor, myogenin, and the late differentiation marker, myosin heavy chain, were detected in L6/S100B myoblasts compared with L6/Mock myoblasts. Interestingly, no obvious changes in the extent of activation of p38 MAPK, a kinase shown to be crucial for myoblast terminal differentiation, could be seen in L6/S100B myoblasts under differentiation conditions. Also, transfection with MKK6EE, a constitutively active mutant of the p38 MAPK upstream kinase, MKK6, while stimulating the extent of myogenin expression and myotube formation in L6/Mock myoblasts as expected, failed to counteract the inhibitory effect of S100B overexpression on these two parameters in L6/S100B myoblasts. These data suggested that S100B might interfere with myoblast differentiation by acting downstream of p38 MAPK, likely by blocking myoblast escape from the cell cycle.

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

MOLECULAR MECHANISM OF S100B-DEPENDENT INHIBITION OF MYOBLAST DIFFERENTIATION

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Myogenesis is an ordered process in which the precursors of skeletal muscle cells, the myoblasts, first proliferate to attain a critical density and then stop proliferating and differentiate into fusion-competent elements that form multinucleated cells, the myotubes, in which contractile proteins assemble to form sarcomeres (1). Proliferation arrest is a critical step in the process of myoblast differentiation in vitro. Factors and/or conditions that favor cell proliferation interfere with myogenic differentiation, which is contrariwise favored by factors and/or conditions that reduce cell proliferation. For example, relatively high serum concentrations, basic fibroblast growth factor and transforming growth factor β stimulate myoblast proliferation thus inhibiting myoblast differentiation via interaction with specific receptors, while low serum concentrations, insulin and insulin-like growth factors and their receptors stimulate myoblast differentiation. In general, factors and/or conditions that activate the Ras/MEK/ERK1-2 signaling pathway stimulate myoblast proliferation, while factors and/or conditions that persistently activate p38 MAPK and/or Akt favor myoblast differentiation. We have recently reported that the Ca^{2+}-modulated protein of the EF-hand type, S100B, inhibits rat L6 myoblast differentiation by interacting with an unknown receptor with a high affinity (K_d ~40 pM) and causing inactivation of p38 MAPK (2) and counteracts the promyogenic effect of RAGE activation by amphoterin (3). We show here that the S100B's ability to inhibit myoblast differentiation depends on activation of the Ras/MEK/ERK1-2 pathway as S100B is unable to inhibit myogenesis in the presence of the MEK inhibitor, PD98059, or in myoblasts transfected with a dominant-negative mutant of Ras. Also, S100B stimulates NF-κB transcriptional activity dose-dependently up to 200 pM, this latter effect being negated in the presence of PD98059, NF-κB inhibitors (PDTC or the NF-κB super-repressor, I-κBαSR) or the antioxidant N-acetylcysteine. Yet, no obvious correlation could be ascertained between S100B effects on NF-κB activity and the S100B inhibitory effect on myogenesis. Also, S100B is unable to stimulate NF-κB transcriptional activity at >200 pM, suggesting that S100B mainly inhibits myogenesis via inactivation of p38 MAPK likely through persistent stimulation of Ras/MEK/ERK1-2 (4). Finally, extracellular S100B inhibits the induction of the anti-proliferative factor, $p21^{WAF1}$, the activity of which is required for myoblasts to exit from the cell cycle (5). Analyses are in progress to identify the factor(s) linking inhibition of p38 MAPK to inhibition of $p21^{WAF1}$ induction in myoblasts exposed to pM doses of S100B under differentiation conditions, and functional correlates of stimulation of NF-κB transcriptional activity by low pM S100B.

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Keywords: S100B, myoblast, proliferation, differentiation, Ras, NF-κB.

DISSECTION OF FOLDING AND UNFOLDING PATHWAYS OF HUMAN S100B

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The S100 proteins are a group of calcium-signalling proteins implicated in such diverse processes as cytoskeletal protein polymerization, cell growth and membrane assembly. Biological and structural studies have shown that calcium-binding to different S100 proteins causes a conformational change in the proteins allowing them to interact with specific target proteins and providing unique biological responses.

One of the hallmarks of the S100 proteins, that distinguishes them from other EF-hand proteins, is their propensity for dimerization. Based on several three-dimensional structures determined using NMR spectroscopy and x-ray crystallographic techniques it has been shown that the dimerization interface is primarily composed of helices I and IV from each monomeric subunit. These helices form an X-type bundle such that helices I and I' run nearly anti-parallel to each other and helices IV and IV' (also anti-parallel to each other) lie nearly perpendicular to each other. The structure of this interface is largely conserved in both the apo- and calcium-bound states.

In an effort to determine the importance of interfacial residues and to understand how the S100 proteins fold into their dimeric structures we have examined the unfolding stability of a series of mutations at the interface of the S100 protein S100B. Three types of mutations have been introduced; (1) single site mutations designed to decrease the stability of the native dimer, (2) truncation mutants designed to identify regions that perturb dimer formation, and (3) single site Trp mutations that allow local changes in the dimer interface to be established. S100B and its mutant forms were examined using fluorescence and NMR spectroscopies to identify the unfolding pathway for the proteins and the differences in stability that each mutant offered.

CHARACTERISATION OF THE HEART OF S100A1-DEFICIENT MICE BY RNA IN SITU HYBRIDIZATION AND IMMUNOSTAINING

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S100 proteins constitute a family of calcium (Ca2+)-binding proteins that exhibit cell- and tissue-specific expression. Protein S100A1 is predominantly expressed in the heart. During embryonic mouse development it has been shown by RNA in situ hybridization that S100A1 expression occurs at equal levels in ventricles and atria early in development (embryonic day 8) and shifts to low levels in the atrial and high levels in the ventricular myocardium during later developmental stages (embryonic day 17.5) [1]. Using RNA in situ hybridization and immunostaining on adult mouse heart sections we are characterising genes that co-express and proteins that co-localise with S100A1. In order to identify factors associated with S100A1 function we use a mouse model deficient in S100A1 expression. Comparisons with the staining pattern of heart sections from S100A1-deficient mice will reveal gene expression/protein localisation differences that are due to the lack of S100A1 and provide insight into the role of S100A1 in the myocardium.

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Keywords : S100A1, heart, ventricle, in situ hybridization, immunostaining, knock out mice

EPIDERMAL GROWTH FACTOR INDUCES S100A14 IN HUMAN LUNG AND BREAST TUMOUR CELLS VIA THE EXTRACELLULAR SIGNAL-REGULATED KINASE CASCADE

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S100A14 is a recently identified gene of the S100 family of calcium-binding proteins which is differentially expressed in human tumours and whose function and regulation remains unknown. To elucidate mechanisms for enhanced S100A14 expression in human breast and lung tumours and establish its clinical significance, we studied the effects of growth factors on its expression as well as regulation of the gene both at the promoter and transcriptional level. S100A14 expression was determined by immunohistochemistry in 107 breast and 120 lung carcinomas. The signalling pathways that target S100A14 expression in response to growth factor stimulation were characterized using various inhibitors of the MAPK signal transduction cascades. S100A14 promoter constructs were generated and used to characterize the gene regulatory regions.

Elevated expression of S100A14 was observed in 69% of breast and 68% of lung tumours. Moreover, the high levels of S100A14 protein correlated with erbB2/HER2 overexpression in breast tumours (p = 0,04). 82,9% of erbB2-positive tumours overexpressed S100A14. We also found an association between high levels of S100A14 and high proliferation index of breast tumours (p = 0,03).

Cell treatment with EGF and TGF-_ resulted in an increase of S100A14 transcripts. Pre-treatment of growth-arrested BET-1A immortalized bronchial epithelial cells with mitogen-activated protein (MAP) kinase inhibitor U0126, blocked the induction of S100A14 by EGF. The EGF-induced S100A14 expression was also inhibited by AG1478, a selective inhibitor of EGF receptor tyrosine kinase activity.

We demonstrate that S100A14 is a target of EGF receptor signalling in lung and breast tumour cells via a pathway involving extracellular signal-regulated kinases 1/2 (ERK1/2) and that levels of erbB2 and S100A14 are tightly correlated in samples of primary breast tumours. Finally, we demonstrate that EGF-dependent S100A14 induction in vitro can be blocked using the MAPK and EGF receptor inhibitors.

These data identify an EGF receptor-driven gene that may provide a novel target for therapeutic intervention in breast and lung cancer.

Keywords : S100, S100A14, EGF, ERK, MAPK, erbB2

THE CRYSTAL STRUCTURE OF HUMAN S100B REVEALS A NOVEL OCTAMERIC ASSEMBLY; IMPLICATIONS FOR RAGE SIGNALLING

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S100B is a member of the EF-hand Ca^{2+}-binding S100 protein family [1]. In addition to its intracellular functions as a regulatory Ca^{2+} sensor protein, S100B is also secreted to the extracellular space where it acts in a cytokine like manner, e.g. stimulating neurite outgrowth. The extracellular action of S100B in the CNS is mediated by the Receptor for Advanced Glycation End products (RAGE) [2] Alternative RAGE independent pathways must be present in different cell types [3,4]. We crystallized human recombinant S100B in the presence of Ca^{2+} and determined the structure at 2.0 Angstrom resolution. The structure revealed an octameric assembly with a tight packing of four homodimers. However the packing is different from the hexameric structure of S100A12 [5]. The contact sites between the dimers exhibit a number of hydrophobic and polar interactions indicating that such multimers should be stable in solution. Indeed we were able to isolate multimeric forms of recombinant S100B, which are fully functional as shown by CD spectroscopy and Ca^{2+}-dependent conformational changes. Interaction studies between the different forms of S100B and a soluble extracellular fragment of RAGE showed that dimeric as well multimeric forms are able to interact with RAGE. The multimeric forms showed a significantly better binding than the homodimeric form. We propose that the presented structure represents the biological active form S100B in RAGE signalling.

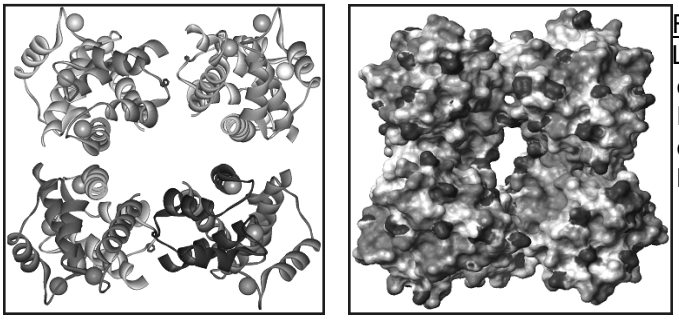

Figure Crystal structure of human S100B
Left side: Ribbon representation of S100B octamer
Right side: Surface representation of S100B octamer, showing the tight interaction between the homodimers.

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Keywords : S100B, X-ray, structure, RAGE

Rab3D AND ANNEXIN A2 PLAY A ROLE IN THE HISTAMINE-EVOKED SECRETION OF vWF FROM HUMAN ENDOTHELIAL CELLS

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Von-Willebrand factor (vWF) and tissue-type plasminogen activator (tPA) are products of endothelial cells that are acutely released into the vasculature following cell activation. The factors exhibit opposing physiological effects, with vWF inducing coagulation and tPA triggering fibrinolysis. Both factors are released after intraendothelial Ca2+-mobilization, but the induced secretion of vWF and tPA reveals subtle differences in calcium requirement, in accordance with findings of vWF and tPA being stored in distinct storage granules within the cells.

In order to identify components that could differentially regulate the release of pro (vWF)- and anti-thrombogenic factors in human endothelial cells we analyzed the contribution of Rab3D and the annexin A2-S100A10 complex, both implicated in exocytotic events in other systems. We show that mutant Rab3D proteins interfere with the formation of bona fide Weibel-Palade bodies (WPbs), the principal storage granules of multimeric vWF, and consequently the acute, histamine-induced release of vWF. In contrast, neither the appearance nor exocytosis of tPA are affected.

We also show that siRNA-mediated down-regulation of annexin A2 and S100A10, in addition to disruption of the complex by microinjection of peptide competitors, result in a marked reduction in vWF but not tPA secretion, without affecting the appearance of WPbs. This indicates that distinct mechanisms underlie the acute secretion of vWF and tPA, thus enabling endothelial cells to fine-regulate the release of thrombogenic and fibrinolytic factors.

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Keywords: calcium/coagulation/exocytosis/fibrinolysis/Weibel-Palade bodies

BIOCHEMICAL CHARACTERIZATION OF ANNEXIN A9 AND IDENTIFICATION OF INTERACTING PROTEINS

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Annexins are a multigene family of evolutionarily conserved proteins characterized by their ability to interact with phospholipids in a Ca^{2+} dependent manner.[1] They are expressed in a variety of cells and tissues and are thought to participate in a number of membrane-related events.

Central to the annexin action is their Ca^{2+} dependent binding to negatively charged phospholipids of cellular membranes. This property is mediated through the structurally highly conserved annexin protein core which has the form of a slightly curved disc and contains two types of Ca^{2+} binding sites, the so-called type II and type III sites. Binding to cellular membranes seems to depend on the integrity of the high affinity type II Ca^{2+} binding sites.

Annexin A9 represents a special annexin subclass insofar as all type II Ca^{2+} binding sites in annexin A9 are inactivated by replacement of the critical amino acid residues.[2] Although annexin A9 is able to bind phosphatidylserine containing vesicles this binding only occures at a Ca^{2+} concentration exceeding 2 mM and appears to be irreversible.[3] These binding characteristics suggest some conformational changes in the annexin A9-Ca^{2+}-liposome complex that may lead to an exposure of Ca^{2+}-insensitive hydrophobic surfaces in the protein. This hydrophobicity increase was further characterized by phase separation using Triton X-114.

In contrast to the highly conserved tetrad annexin core the N-terminal domains of different annexins are highly divergent and are believed to confer specifity to the individual members of the protein family. Although showing limited sequence homology, the N-terminal regions of annexin A2 and A9 share the potential of forming an amphiphatic α-helix. In annexin A2 this helix is essential for binding of the intracellular ligand S100A10. Therefore we started to identify possible binding partners of human annexin A9 by using an affinity chromatography approach and subsequent MALDI-MS analysis. Results of these approaches will be discussed.

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Keywords : acidic phospholipids, membrane binding, Triton X-114 phase separation, Ca^{2+}-insensitive annexins

THE PROPER POSITIONING AND MORPHOLOGY OF RECYCLING ENDOSOMES IS AFFECTED BY ANNEXIN A2/S100A10-DOWN-REGULATION

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Annexins are a family of cytosolic Ca^{2+} binding proteins characterized by their reversible Ca^{2+} dependent association with intracellular membranes. Each annexin comprises a highly conserved protein core which harbours the Ca^{2+} and phospholipid binding sites and the N-terminal region which is unique for a given annexin.[1]
Annexin A2, which resides in a tight heterotetrameric complex with the S100 protein S100A10 (p11), has been implicated in the structural organisation and dynamics of endosomal membranes.

Using small interfering RNA (siRNA) we achieved significant and specific reduction of annexin A2 and S100A10. This did not affect the uptake of receptors or fluid phase tracers into sorting endosomes and did not change the routing to the late endocytic pathway. However, the positioning and morphology of recycling endosomes, positive for rab11, is significantly altered in the annexin A2-negative cells.[2] Molecules which are transported through the recycling endosomes accumulated in this compartment and tubules of the recycling compartment appeared bent and ring-shaped. Effects on molecules trafficking through recycling endosomes will be discussed.

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Keywords: endocytosis, membrane traffic, membrane organization, RNA interference

ANNEXIN 2 PROMOTES THE FORMATION OF LIPID MICRODOMAINS REQUIRED FOR CALCIUM-REGULATED EXOCYTOSIS OF DENSE-CORE VESICLES

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Annexin 2 is a calcium and lipid-binding protein present in cells either as a 36-kD monomer (p36) or as a 90-kD tetramer containing 2 copies of p36 and 2 copies of the S100 protein p11. Annexin 2 has been implicated in a number of membrane-related events particularly in calcium-regulated exocytosis. We previously reported that catecholamine secretion requires the translocation and formation of the annexin 2 tetramer near the exocytotic sites (1). To obtain direct evidence for a role of annexin 2 in exocytosis, we have modified its expression level in chromaffin cells by means of the Semliki Forest virus expression system. Using a real-time assay for individual cells, we found that the reduction of cytosolic annexin 2, and the consequent decrease of annexin 2 tetramer at the cell periphery, strongly inhibited exocytosis, most likely at an early stage prior to membrane fusion. Secretion was also severely impaired in cells expressing a p36/p11 XM chimera, which aggregates cytosolic annexin 2 and prevents its translocation to the cell periphery (2).

Annexin 2 has been recently described as a calcium-dependent promoter of lipid microdomains (DRM) in the plasma membrane (3). To investigate the possibility that annexin 2 participates to the organization of DRMs required for exocytosis in neuroendocrine cells, we examined the association of p36 and p11 with chromaffin cell lipid microdomains purified on density gradients. We found that both p36 and p11 floated with DRMs extracted from calcium-stimulated cells. The functional importance of the binding of annexin 2 to DRMs was investigated in chromaffin cells expressing the XM chimera. Using fluorescent cholera toxin combined with confocal laser scanning microscopy and amperometry, we found that the reduction of functional p36 at the cell periphery inhibited in parallel exocytosis and the formation of ganglioside GM1-containing lipid rafts at the plasma membrane. This suggests the involvement of annexin 2 in the formation and/or stabilization of DRMs during exocytosis. One attractive speculation is that the translocation of annexin 2 to the plasma membrane, by favoring the local formation of lipid microdomains, brings a new configuration of membrane-bound constituents at the exocytotic sites with potentially important consequences for the subsequent docking and fusion events.

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Keywords : Annexin2 - Chromaffin cells – Exocytosis- Lipids microdomains-

CHARACTERIZATION OF SINGLE-TRYPTOPHAN MUTANTS OF HUMAN ANNEXIN A6 ISOFORM 1: EVIDENCE FOR THE ROLE OF TRP343 IN GTP BINDING BY THE ANNEXIN

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The 8-repeat domain annexin A6 (AnxA6) is the largest member of the annexin family of calcium- and membrane-binding proteins expressed in human organism. Accumulated experimental evidence indicated that AnxA6 may bind GTP *in vitro*, although the localization of the nucleotide-binding domain within the annexin molecule has not been established for certainty. Recently, GTP-induced voltage-dependent ion channel activity of AnxA6 at pH 7.4 was observed [1]. In addition, molecular simulations revealed a nucleotide-binding site in the middle part of the protein, distributed in two distinct domains located at 291-299 and at 640-648 residues. The consensus sequence present in other human annexins suggests that the binding site in AnxA6 is not accidental [2]. Several amino acid residues are located below the distance of 4 Å from the nucleotide-binding site, among them W343. To further characterize GTP-binding site of AnxA6 we used site directed mutagenesis technique and found that mutant W343S but not W192S neither binds GTP nor is able to form voltage-dependent ion channels in planar lipid membranes. We suggest that GTP-binding properties of AnxA6 are crucial in mineralization process that plays a major role during the development of skeletal tissues. Despite its obvious importance, little is known about its regulation. It has been demonstrated by Wang & Kirsch [3] that terminal differentiation and mineralization of cultured growth plate chondrocytes is accompanied by an increase in cytosolic Ca^{2+} concentration, followed by up-regulation of AnxA2, A5 and A6 gene expression, and release of matrix vesicles (MVs) containing annexins and alkaline phosphatase. This suggested implication of specific annexin isoforms in mineralization process. Furthermore, it was proposed by Arispe *et al.* [4] that GTP modulated calcium channels formed by annexin molecules in the membrane of MVs are responsible for the Ca^{2+} entry into lumen of these extracellular organelles. The GTP-induced ion channel activity of AnxA6 was found associated with the structural flexibility of the protein characterized by changes in secondary and tertiary structure of AnxA6, suggesting that such structural flexibility could contribute to a molecular mechanism of AnxA6 acting as an intracellular GTP biosensor in biomineralization or an atypical G protein, responsible for detecting the Ca^{2+}/GTP signals in chondrocytes. As an alternative, it can be speculated that AnxA6/nucleotide binding participates directly or indirectly in the regulation of functions of other GTP-binding or GTPase-activating proteins and, therefore, in modulation of intracellular signalling patways mediated by these proteins.

This work is supported by Grant No. 3 PO4A 007 22 from the Polish Committee for Scientific Research.

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Key words: annexin A6; nucleotide-binding domain; ion channel; biomineralization

LATE ABSTRACTS

ANNEXIN A2 BINDS ITS COGNATE mRNA WITH HIGH AFFINITY

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The multifunctional protein annexin A2 (anxA2) is a member of the annexin superfamily of calcium- and phospholipid-binding proteins. We have previously shown that a fraction of anxA2 is associated with a specific subpopulation of mRNPs (1). AnxA2 interacts directly with both the 5'- and 3' untranslated regions of *anx*A2 mRNA with high specificity, but not to its coding region. Higher order structures of the mRNAs, like stem-loop structures, rather than short sequence motifs alone are involved in the binding. The surface plasmon resonance (SPR) technique was used to measure the affinity of this binding. The interaction is of high affinity, since full-length anxA2 mRNA binds to immobilised recombinant anxA2 with a K_D in the low nM range, while the K_D for the interaction of the 3'UTR of anxA2 is several fold higher, although still in the nM range. The binding of heparin to immobilised anxA2 was highly increased in the presence of calcium and magnesium, and the effect of these divalent cations on binding of mRNA to anxA2 is currently investigated. Preliminary results were obtained using a recombinant MBP-anxA2. We are now producing a recombinant HisMBP-anxA2, which after cleavage with HisTEV, is quality testet by biophysical methods. This purified anxA2 is less prone to aggregation than the previous recombinant anxA2.

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Keywords: annexin A2, mRNA-binding protein, surface plasmon resonance

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