cADPR and NAADP signalling
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SA40
Real-time imaging of cAMP using a FRET-based biosensor
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A key feature of signal transduction is the convergence of a very large variety of extracellular stimuli onto an extremely limited number of intracellular second messengers. By transducing inside the cell the highly variable and complex extracellular information, second messengers can tightly and specifically modulate a disparate variety of intracellular functions and a signalling code based simply on messenger concentration grading appears insufficient to ensure the necessary specificity and diversity to signalling. In the last decade much attention has thus been focused on the spatial and temporal dynamics of second messengers and it has become clear that targeting and compartmentation of signalling enzymes and effectors close to their activators and targets is crucial to ensure tight regulation and specificity of action of signalling cascades.

Figure 1. Schematic representation of the FRET-based cAMP sensor.
The cAMP/PKA signalling pathway modulates a large variety of cellular functions, as diverse as growth, movement, metabolism and synaptic plasticity. cAMP signalling relies on the organisation of macromolecular complexes in the generation of which a central role is played by A kinase anchoring proteins (AKAPs). AKAPs can act as multiscaffolding proteins that are targeted to specific subcellular locations and can bind simultaneously the main effector of cAMP, PKA and several other signalling molecules (such as plasma membrane receptors, phosphatases, PKC, etc.). The organization of the cAMP/PKA pathway in such signalling domains is thought to be crucial for achieving specificity of response (Edwards & Scott, 2000) and although the concept of cAMP/PKA compartmentalized signal transduction is largely accepted, the detailed description of how this is operated is still missing and the information on the spatio-temporal dynamics of cAMP is extremely poor. This is mainly due to the lack of methodologies for the analysis of cAMP changes in live cells. We recently generated a sensor for cAMP by genetically linking the R and C subunits of PKA each to a different mutant of GFP (Zaccolo et al. 2000). These mutants (i.e. EYFP and ECFP) show spectral characteristics that make them a suitable pair for fluorescence resonance energy transfer (FRET). FRET is a quantum-physical phenomenon whereby the energy of the excited state of a donor fluorophore (ECFP) is transferred to an acceptor fluorophore (EYFP) that lies in its close proximity (<10nm). Cells transfected with this probe can be analysed at the fluorescence microscope by exciting ECFP only and changes in FRET are conveniently measured as A (emission cyan/emission yellow). This methodology allows fast and reproducible monitoring of [cAMP], changes in single cells with a high spatial (<10µm) and time (<10ms) resolution. By using this sensor we could gain an important insight into the spatio-temporal dynamics of β-adrenergic signalling in heart cells (Zaccolo & Pozzan, 2002). In particular we could show that: (i) β-adrenergic stimulation generates in cardiac myocytes multiple local microdomains of high cAMP in correspondence of the Z band in the sarcomere; (ii) the restricted pools of cAMP show a range of action as small as ~1µm and free diffusion of the second messenger is limited by the activity of phosphodiesterases; (iii) the steep gradients of cAMP specifically activate a subset of protein kinases A anchored in proximity of the Z band.


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PLC-zeta: a sperm protein that triggers calcium oscillations in mammalian eggs
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The activation of egg development by the fertilizing spermatozoon is produced by a rapid rise in cytosolic free Ca2+ concentration in all plant and animal species examined thus far (Stricker, 1999). In mammals, sperm-egg fusion results in a remarkable series of cytosolic Ca2+ oscillations that have been shown to be essential for normal development of the early embryo (Stricker, 1999). This spectacular Ca2+ signalling event arises from transient elevations in inositol 1,4,5-trisphosphate (IP3) levels that cause the activation of IP3 receptor-mediated Ca2+ release from intracellular stores in the egg. However, the precise mechanism describing how the stimulation of IP3 production occurs within the egg following fusion by sperm has remained undefined.
The 'sperm factor' hypothesis is a proposal for the signalling mechanism occurring at fertilization in which spermatozoa release a soluble Ca\(^{2+}\)-releasing factor that enters the egg upon sperm-egg fusion and this 'sperm factor' somehow generates the observed cytosolic Ca\(^{2+}\) oscillations (Swann, 1990). This is in contrast with another proposal, the 'egg receptor' hypothesis (Runft et al. 2002), a mechanism that involves a specific, sperm-surface ligand interacting with a receptor on the egg plasma membrane to trigger activation of IP\(_3\), generation via an egg cytosolic phosphoinositide-specific phospholipase C (PLC). An advantage of the 'egg receptor' hypothesis is that it recruits an established signalling mechanism, well-characterised in somatic cells, whereby hormone ligand interaction with surface receptors triggers a signalling cascade culminating in PLC activation and IP\(_3\), elevation. Conversely, significant empirical evidence supports the 'sperm factor' proposal, as the microinjection of soluble sperm extracts (Swann, 1990), or of a single spermatozoa (Nakano et al. 1997), triggers Ca\(^{2+}\) oscillations in mammalian eggs indistinguishable from those at fertilization, and the cytoplasmic fusion of sperm and egg precedes the first Ca\(^{2+}\) rise (Lawrence et al. 1997). Analysis of mammalian sperm extracts has indicated a high level of PLC activity that coelutes with Ca\(^{2+}\) releasing ability, suggesting a sperm PLC may be a component of the 'sperm factor' (Jones et al. 1998), although it appears not to be a previously identified PLC isoform (Parrington et al. 2002).

We have employed a contemporary strategy whereby novel, PLC-related, short nucleotide sequences present in mammalian testis, were identified in the public domain database of expressed sequence tags (EST) using the heuristic Blast similarity search algorithm. An unique isoform of PLC, termed zeta (PLC\(_{\zeta}\)), was thus identified and characterised from mouse (Saunders et al. 2002) and human and monkey (Cox et al. 2002) sperm. These studies have shown that (a) PLC\(_{\zeta}\) triggers Ca\(^{2+}\) oscillations in mouse eggs indistinguishable from those at fertilization, (b) PLC\(_{\zeta}\) removal from sperm extracts abolished Ca\(^{2+}\) release in eggs, (c) the PLC\(_{\zeta}\) content of a single sperm was sufficient to produce Ca\(^{2+}\) oscillations as well as normal embryo development to blastocyst. Our results are consistent with sperm PLC\(_{\zeta}\) as a component of the physiological trigger for development of a fertilized egg into an embryo.

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SA42

Calcium dynamics during mammalian egg activation: is there a developmental impact?

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In the free calcium concentration of the egg cytoplasm sperm triggers a series of repetitive spikes at fertilization that are responsible for activation. The interest of this very short developmental period resides in three particular aspects. First of all, it is well established that the processes of oocyte activation co-ordinate a complex epigenetical chain of events resulting in the remodelling of the parental genomes. Secondly this initial biochemical activity of the egg cytoplasm has the unique property of 'reprogramming' a somatic nucleus when it is exposed to its environment. The third aspect resides in the fact this global and very complex molecular dynamics of the oocyte are triggered and paced by a repetitive Ca\(^{2+}\) signal that is comparatively simple and lasts a very short period of time.

While considerable knowledge on how sperm triggers calcium oscillations has recently been produced, still the extreme variability in signal kinetics between eggs, i.e. amplitude, number and frequency, usually observed after fertilization, makes it practically impossible to run meaningful experiments on the exact functions and biological impacts of signal parameters.

Since this calcium dynamic orchestrates the many changes through which a single fertilized egg cell turns into a complex organism, taking over the control of this process might give us the possibility of graduating and driving the early developmental events in order to establish correlations between early and late developmental events.

Several approaches can be used to stimulate or bypass the natural mechanisms that cause repetitive Ca\(^{2+}\) increase. Our initial approach uses electropermoabilisation to create a Ca\(^{2+}\) influx into the cell to either reproduce the natural signal on non-fertilized oocytes that are not capable of releasing Ca\(^{2+}\) from intracellular stores, or to stimulate the calcium-induced calcium release processes in fertilized eggs. This technique is simple and very common but it has a dramatic impact on the cell when the electrical current generated by the electrical pulse is too high. In fact, the eggs usually die after repetitive electrical stimulation.

Since the impact depends on the external ionic concentration we have developed a microfluidic processor that assures us of fast washing before the electrical pulse to minimise the ionic conductivity of the media before the pulse and a very fast washing after the pulse to block the calcium influx by facilitating membrane healing. This microfluidic artifice associated with transient electropermoabilisation of the membrane gives us good control over the amplitude and frequency of the signal in an entire oocyte population, thus assuring standardization and reproducibility.

Using this technology, we have produced several pieces of information. Firstly, the calcium stimulus is the most efficient signal activating mammalian eggs when it is applied in a repetitive manner. Secondly, repetitive Ca\(^{2+}\) signals drive the onset and progression of a series of cellular and biochemical events that characterize fertilization, i.e. cortical granules exocytosis, cell cycle resumption with concomitant decrease in MPF and MAP kinases activities, recruitment of maternal mRNAs differently. The Ca\(^{2+}\) regimen appears to affect the methylation pattern of several DNA segments but the dynamics of early cleavage does not appear to be determined by either the frequency or the amplitude of the calcium signal. Thirdly, in the
rabbit species, amplitude and temporal modulation of the Ca$^{2+}$ signals during the early minutes of oocyte activation impacts the extent of developmental organization and differentiation at the post-implantation stages of parthenogenetic embryos.

On the basis of these results, artificial control of the Ca$^{2+}$ dynamic during oocyte activation leads us to several important deductions. First of all, oocytes have the potential to decode even minute levels of perturbation during the process of activation. Secondly, some post-implantation developmental variations are caused by changes in the regime of calcium signalling. Thirdly, by assuring the fidelity of the Ca$^{2+}$ response, new approaches are opened to determining the biochemical mechanisms driving the egg-to-embryo transition, thus making it possible to better understand hidden epigenetic regulation. In addition, such an approach improves developmental predictions in applied biotechnology.

By refining our technology we hope to be able to drive the calcium dynamic in fertilized eggs in order to establish a link between early and late developmental events until term to assist the process of fertilization.

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SA43
An exploration of the calcium signalling during somitogenesis in zebrafish
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During vertebrate embryonic development, somites are formed from the unsegmented presomitic mesoderm (PSM) by a highly regulated process. They are formed in pairs in an anterior-to-posterior progression within the PSM, on either side of the notochord and are transient structures, which ultimately go on to form the axial skeleton, the dermis and the skeletal musculature. Using f-aequorin (a Ca$^{2+}$-sensitive bioluminescent reporter; Shimomura et al. 1990) and an ultrasensitive photon-imaging microscope (Webb et al. 1997), we have begun to: (1) visualize and characterize the Ca$^{2+}$ signals that occur in the trunk during the segmentation period; (2) to explore the developmental significance and function of the Ca$^{2+}$ signals; and (3) to explore the source and release mechanism of the Ca$^{2+}$ generating the signals.

Imaging data indicate that localized Ca$^{2+}$ elevations are generated within the PSM before the somites are formed, within specific regions of the maturing somites after they have formed and within the notochord. Our analysis so far has concentrated on the Ca$^{2+}$ transients generated by the maturing somites after they have formed. These transients were not seen in every embryo examined (n = 15) in a regular, repeating manner (i.e. on either side of the notochord within every pair of formed somites). However, although appearing to be chaotic, when they did occur, all the transients appeared at the same location with respect to somite morphology (i.e. at the medial and lateral extremities of the somites), and were of similar amplitude, duration, and involved approximately the same number of cells. In order to explore the significance and function of these seemingly chaotic signals, we loaded embryos with either photolabile caged Ca$^{2+}$ or caged Ca$^{2+}$ buffer, then illuminated specific regions of formed somites. The precocious elevation of Ca$^{2+}$ in embryos loaded with caged Ca$^{2+}$ (NP-EGTA) caused the formation of shorter somites along the medio-lateral axis, compared with untreated controls. Moreover, when regions of active buffer were generated by localized illumination of embryos loaded with the caged Ca$^{2+}$ chelator, diazo-2, this resulted in the formation of elongated somites along the medio-lateral axis, compared with the untreated controls.

We suggest therefore, that these seemingly chaotic Ca$^{2+}$ transients play a role in defining the medio-lateral boundaries of the maturing somites. As they do not occur in a regular, predictable manner, we propose that they are not the primary signal that defines these boundary limits, but are generated only when required. They act, therefore, to reinforce the primary medio-lateral boundary definition signal only in situations where the primary signal is ineffective.

It has been reported that Ca$^{2+}$ transients observed during somitogenesis in Xenopus originate from both cADPR receptor and IP$_3$ receptor-activated Ca$^{2+}$ stores (Ferrari & Spitzer, 1999), rather than from Ca$^{2+}$ entry via extracellular sources. When f-aequorin-loaded zebrfish embryos were incubated in Ca$^{2+}$-free medium containing EGTA during the segmentation period, Ca$^{2+}$ transients were still visualized in the trunk, and somitogenesis occurred normally for a while before the embryos dissociated. Thus these data suggest that zebrafish are similar to Xenopus where the source of Ca$^{2+}$ during somitogenesis is intracellular rather than extracellular. However, while treating embryos with U73122 (a phospholipase C blocker) resulted in medio-laterally elongated somites, ryanodine (a cADPR receptor antagonist) had no effect on somite morphology. Thus these results suggest that unlike Xenopus, in zebrafish Ca$^{2+}$ is released through IP$_3$ receptor-activated stores alone during early somite maturation. Furthermore, we have demonstrated the localization of IP$_3$ receptors within the maturing somites via immunohistochemistry and have shown that they are functional by visualizing intracellular Ca$^{2+}$ release induced by localized photo-activation of caged IP$_3$.


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SA44
Imaging PIP$_2$ dynamics and PKC activation at fertilization
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Activation of embryonic development at fertilization is known to be triggered by long-lasting Ca$^{2+}$ oscillations in mammalian eggs. These sperm-induced Ca$^{2+}$ transients start as propagated waves originating in the egg’s cortex. Involvement of the inositol trisphosphate (InsP$_3$) receptors in this process has been demonstrated, suggesting that fertilization activates phosphoinositide metabolism (Carroll, 2001). Furthermore, a novel sperm-specific phospholipase C isofrom – PLCzeta – has recently been demonstrated to mimic sperm-induced egg activation when microinjected into mammalian eggs (Saunders et al. 2002). Fertilization is thus considered to activate primarily phosphatidylinositol 4,5-bisphosphate (PIP$_2$) hydrolysis to
generate InsP₃ and diacylglycerol (DAG). However, the extent and spatiotemporal pattern of PIP₂ hydrolysis are unknown.

Using molecular tools and confocal microscopy, we investigated the activation of the phosphoinositide pathway at fertilization in mouse eggs. To monitor PIP₂ levels, we microinjected the eggs with an mRNA encoding a GFP-tagged pleckstrin homology domain that selectively binds PIP₂ (PH-GFP). As another indicator of phosphoinositide signalling, we expressed a GFP-tagged conventional protein kinase C (PKCγ-GFP; Oancea & Meyer, 1998). Intracellular Ca²⁺ level was monitored simultaneously using fura-2.

PH-GFP exhibited a strong plasma membrane labelling in mouse eggs, with accumulation of the fusion protein in the microvilli of the vegetal pole. The specificity toward PIP₂ labelling was assessed by expressing a mutant version of PH-GFP that does not recognize PIP₂. At fertilization, we did not detect any significant loss of plasma membrane staining that could indicate PIP₂ hydrolysis. Rather, a net increase in PIP₂ labelling was observed. This increase in plasma membrane PIP₂ was transient, activated by the rise in cytoplasmic Ca²⁺ and could be mimicked by photorelease of caged InsP₃. In contrast, when using ionomycin to activate PIP₂ hydrolysis, a dramatic loss of PIP₂ labelling was observed. Two toxins that inhibit cortical granule release at fertilization, laspakolin and Botulinum neurotoxin A, had inhibitory effects on the rise in PIP₂, suggesting that this increase in plasma membrane PIP₂ may play a role in exocytosis of the cortical granules (Halet et al. 2002).

PKCγ-GFP was found to rapidly and reversibly translocate to the plasma membrane in a manner that mirrored Ca²⁺ release at fertilization. To investigate the role of Ca²⁺ and DAG in PKC translocation, we expressed GFP fusion constructs of the isolated C1 or C2 domains of PKCγ which bind DAG or Ca²⁺, respectively. Unexpectedly, no significant translocation of C1-GFP was observed at fertilization, despite efficient translocation after stimulation with a phorbol ester or an analogue of DAG. Also, ionomycin-induced PIP₂ hydrolysis generated DAG that recruited C1-GFP to the plasma membrane. In contrast, the dynamics of C2-GFP at fertilization closely matched the dynamics of the full-length PKCγ-GFP.

These data suggest that the physiological Ca²⁺ signalling pathway at fertilization does not lead to significant PIP₂ hydrolysis or DAG accumulation in the plasma membrane. In addition, PKC translocation seems to be mediated solely via Ca²⁺ binding to the C2 domain.


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SA45

PITP proteins as lipid sensors: transfer function regulated by EGF

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Phosphatidylinositol transfer proteins (PITPs) bind one molecule of either phosphatidylinositol (PI) or phosphatidylcholine (PC) and can mediate their transfer between membrane compartments in vitro. The best-characterised mammalian PITPs are PITPa and PITPβ, two highly homologous proteins encoded by distinct genes. Genetic studies indicate that PITPβ is an essential gene, as ablation of the gene is embryonically lethal, whilst ablation of PITPa leads to the birth of live mice which only survive for a couple of weeks (Hamilton et al. 1997; Alb et al. 2002). Biochemical studies involving reconstitution of cytosol-depleted cell preparations have demonstrated the requirement of PITPa and -β in signal transduction and in membrane traffic. From such analysis, a requirement for PITP has been identified in phospholipase C (PLC)-mediated phosphatidylinositol(4,5) bisphosphate (PI(4,5)P₂) hydrolysis, in the synthesis of 3-phosphorylated lipids by phosphoinositide 3-kinases, in regulated exocytosis and in the biogenesis of vesicles at the Golgi. Studies aimed at elucidating the mechanism of action of PITP in each of these seemingly disparate functions have yielded a singular theme; the activity of PITP stems from its ability to transfer PI from its site of synthesis to sites of cellular activity and to stimulate the local synthesis of phosphorylated forms of PI including PI(4)P, PI(4,5)P₂, PI(3)P and PI(3,4,5)P₃ by delivering PI to the lipid kinases involved in phosphoinositide synthesis (Hsuan & Cockcroft, 2001). Using FLIM (fluorescence lifetime imaging microscopy) to measure FRET (fluorescence resonance energy transfer) between GFP-PITP proteins and fluorescently labelled phospholipids, we report that PITPa and PITPβ can dynamically interact with PI or PC at the plasma membrane only when stimulated with epidermal growth factor (EGF). In addition, PITPβ is localized at the Golgi, and upon EGF addition, the lipid environment is altered, resulting in enhanced FRET (B. Larijani et al. 2002, submitted). Our observations demonstrate that the transfer function of PITPa and PITPβ is a regulated process involving dynamic behaviour in vivo. Previous studies have shown that both PITPa and PITPβ can reconstitute PLC signalling (Cunningham et al. 1996) and the observation that both PITPa and PITPβ are found at the plasma membrane following stimulation emphasises that PITP proteins have overlapping functions in PLC signalling.


SA46

Feedback regulation of IP₃-mediated Ca²⁺ signalling

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Ca²⁺ release from intracellular stores via the inositol 1,4,5-trisphosphate receptor (IP₃R) regulates a vast array of important cell functions including smooth muscle contraction, secretion, immunity, fertilization and synaptic plasticity. The versatility of Ca²⁺ signals is thought to be based on the complexity of signals that can be coded by the release of Ca²⁺ from the intracellular stores, i.e. Ca²⁺ release may be localised and transient (Ca²⁺ puffs), may propagate regeneratively across a cell (Ca²⁺ waves) and may be repeated periodically (Ca²⁺ oscillations). These Ca²⁺ signals convey distinct information depending on their spatiotemporal patterns. The dynamic changes in the intracellular Ca²⁺ concentration ([Ca²⁺]) are thought to require the presence of certain forms of feedback mechanism during Ca²⁺ mobilisation. Interestingly, IP₃R activities are sensitive to [Ca²⁺], at submicromolar to micromolar concentrations in a biphasic manner. Therefore, it is possible that the Ca²⁺-mediated feedback regulation of Ca²⁺ release contributes to the generation of...
spatiotemporal dynamics of Ca$^{2+}$ signals. We identified the Ca$^{2+}$ sensor region of the IP$_3$R, and the substitution of glutamate at position 2100 of type 1 IP$_3$R by aspartate resulted in a 10-fold decrease in Ca$^{2+}$ sensitivity (Miyakawa, 2001). In agonist-stimulated cells expressing the low-Ca$^{2+}$ sensitivity mutant IP$_3$R, the rates of increase in [Ca$^{2+}$]$_i$ were markedly reduced and Ca$^{2+}$ oscillations were abolished. These results indicate that the Ca$^{2+}$-mediated feedback regulation of IP$_3$R is important for the generation of spatiotemporal patterns of Ca$^{2+}$ signals. Another candidate molecule that may be under a Ca$^{2+}$-mediated feedback regulation is IP$_3$. We succeeded in generating an IP$_3$ indicator based on the pleckstrin homology domain of phospholipase C-$\delta$1 (Hirose et al. 1999). Using this new method, we showed that the IP$_3$ production is also dependent on [Ca$^{2+}$]$_i$, and indeed we found IP$_3$ oscillations that were synchronizing with Ca$^{2+}$ oscillations. Furthermore, we showed that the depolarisation-mediated influx of Ca$^{2+}$ induces IP$_3$ production in cerebellar Purkinje cells (Okubo et al. 2001). These results demonstrate that both IP$_3$ production and Ca$^{2+}$ release are under the feedback control of [Ca$^{2+}$]$_i$, which is an important molecular basis of the complex spatiotemporal patterns of Ca$^{2+}$ signals.