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Calcium signals mediated by IP_3-gated channels in endothelial cells in pre-capillary arterioles

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Although localized Ca^{2+}-release signals (puffs) arising from the opening of IP_3 channels on the sarcoplasmic reticulum (SR) have been described in endothelial cells, to date there are no descriptions of these events in native cells in intact preparations of terminal arterioles. Given the expected importance of cell to cell contact, and interactions with neighbouring smooth muscle cells in modulating local Ca^{2+} signals and propagated Ca^{2+} waves, we have characterized these events.

Fluo-4 was loaded into *in situ* endothelial cells in pre-capillary arterioles contained in small bundles of rat ureter. Both spontaneous and agonist-induced events were recorded using wide-field, real time confocal imaging. Both spontaneous and carbachol–induced Ca^{2+} signals in the endothelial cells were resistant to Ca^{2+}-free (2mM EGTA) solutions, nifedipine and ryanodine (n=17). In contrast, the Ca^{2+} events were completely and reversibly blocked by cyclopiazonic acid (Ca^{2+}-ATPase inhibitor), 2-APB (IP_3Rs antagonist) or U73122 (phospholipase C inhibitor) (n=11). There was significant variability in frequency, amplitude, time course and spatial spread of Ca^{2+} puffs not only among different discharging sites but also for the same site. These data suggest that IP_3-gated channels in the endothelial cells exist in clusters containing variable numbers of channels and that a variable number of channels can be recruited within these clusters. The number of discharging sites per cell varied between 2-3 (n=30). The main discharging site, which showed the highest frequency of Ca^{2+} puffs (0.33±0.06 Hz, n=22 sites), was normally located at the edge of the cell membrane adjacent to a smooth muscle cell in the thicker part of the endothelial cell, several microns away from the nucleus. Additional discharging sites were located in the distal narrow regions of the cell. The frequency, amplitude, time course and spatial spread of Ca^{2+} puffs as well as the number of the discharging sites per cell were increased in the presence of low concentrations of carbachol (50-100 nM) (n=27). Higher concentrations of carbachol (1-10 μM) induced oscillatory propagating Ca^{2+} waves that originated from several sites (n=27). These Ca^{2+} waves could have different orientation and spatial spread and very often when initiated at distal ends of the cells at the same time could annihilate each other when reaching central parts of the cell.

These data suggest that in the endothelial cells propagating Ca^{2+} waves are mediated exclusively by IP_3 receptor gated channels which can be spatially recruited to induce a regenerative Ca^{2+} wave.

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Regulation of inositol 1,4,5-trisphosphate receptors ($InsP_3R$) by protein kinase B

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$InsP_3$-induced Ca^{2+} signals regulate many cellular processes from fertilisation to cell death. In addition to regulation by $InsP_3$ and Ca^{2+}, $InsP_3R$ activity is modulated by protein-protein interactions and post-translational modifications. Using the Scansite motif-searching algorithm (1) to interrogate the $InsP_3R1$ sequence for consensus sites for protein phosphorylation, we have identified a consensus site for phosphorylation by protein kinase B (PKB/Akt) at its COOH-terminus. PKB is a serine/threonine kinase that is recruited to the plasma membrane upon growth factor/agonist stimulation where it subsequently activated. A number of substrates for PKB have been identified, many of which are important in cell proliferation, cell survival and glucose metabolism. Here, we investigated whether the $InsP_3R$ is a bona-fide substrate for PKB and whether PKB affects $InsP_3$-mediated calcium signals.

To test whether $InsP_3Rs$ were phosphorylated by PKB, $InsP_3Rs$ were immunoprecipitated from HeLa cells that that had been maintained in ^{32}P orthophosphate and in which PKB activity had been modified using pharmacological or molecular approaches. Stimulation of serum-starved HeLa cells with either Insulin (1 μg/ml, 5 min) or FBS (10% v/v) resulted in an LY294002 (10 μM) sensitive phosphorylation of $InsP_3Rs$. No phosphorylation was detected in non-stimulated cells. Furthermore, $InsP_3Rs$ isolated from HeLa cells grown under serum replete conditions were also phosphorylated in an LY294002-sensitive manner (n=3). By immunoprecipitation of overexpressed $InsP_3R1$, in which the candidate phosphorylatable serine was mutated to an alanine, we determined that the predicted PKB consensus site was susceptible to phosphorylation by PKB. The effect of $InsP_3R$ phosphorylation upon $InsP_3$-induced Ca^{2+} release was next investigated in HeLa stable cell lines inducibly overexpressing either constitutively active (CA)-PKB, kinase dead (KD)-PKB or YFP. Experiments were performed on three separate days on three cover slips per day. Statistical significance was determined by ANOVA. Using fura-2 imaging, we found that the percentage of cells responding to 0.5 μM histamine (4.01±2.7%, $p<0.05$) was significantly inhibited by PKB overexpression when compared to cells expressing KD-PKB (42.71±3.73) or YFP (54.18±8.77). This effect was due to a decrease in $InsP_3R$ sensitivity, since the amplitude of the Ca^{2+} signal induced by a cell permeant $InsP_3$-ester were also significantly inhibited by PKB overexpression (404.7±25.54 nM in controls vs 278.3±15.4 nM in CA-PKB expressing cells, $p<0.05$).

Taken together, we have shown a novel role for PKB in regulating calcium signalling via a direct interaction with the $InsP_3R$. We propose that this interaction decreases $InsP_3R$ sensitivity to agonist stimulation and creates a link between growth factor signalling, the $InsP_3R/CaCa^{2+}$-signalling cascade and apoptosis.

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Where applicable, the authors confirm that the experiments described here conform with the Physiological Society ethical requirements.

C82

Inositol 1,4,5-trisphosphate ($InsP_3$)–induced Ca^{2+} release controls cardiac hypertrophy

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As a result of increased haemodynamic load, genetic factors or disease, the heart undergoes a hypertrophic response. Genetic manipulation of components of Ca^{2+} regulated gene transcription pathways has revealed that this ion has a fundamental role in stimulating cardiac hypertrophy. However, it is difficult to reconcile how Ca^{2+}-sensitive gene transcription can be controlled with great fidelity and specificity independent of the increases in Ca^{2+} that occur during every heart beat. Previously, we have shown that endothelin (ET-1) stimulates $InsP_3$-dependent Ca^{2+} release in cardiac myocytes (1). ET-1 is also a potent inducer of cardiac hypertrophy. Here we investigated whether ET-1/$InsP_3$-induced Ca^{2+} release contributes to its pro-hypertrophic action. Experiments were performed using spontaneously contracting primary cultures of neonatal ventricular rat myocytes (NRVM). NRVMs were prepared by collagenase digestion of cardiac ventricles isolated from 1-2 day old Wistar rat pups. Quantitation of atrial natruiretic factor (ANF) by immunofluorescence and real-time PCR, together with cell surface area were used as measures of hypertrophy. For brevity, only the percentage of ANF expressing cells will be presented. All statistics presented are a mean ± SEM of the data generated from myocytes isolated on three or more separate days. Statistical significance was determined by ANOVA and accepted if $p < 0.05$.

To investigate the mechanism of ET-1 induced hypertrophy, 2-APB (10 μM) was first used to inhibit $InsP_3$-induced Ca^{2+} release. 2-APB decreased the percentage of myocytes expressing ANF following ET-1 stimulation (100 nM, 24 hr) from 54.69±2.5% to 29.85±0.84% ($p<0.05$). 17.55±4.33% of control, non-stimulated cells expressed ANF. To further demonstrate the $InsP_3$-dependence of this process, $InsP_3$-induced Ca^{2+} release was inhibited by adenoviral mediated expression of InsP3- 5'phoshatase. In myocytes expressing 5'phosphatase, ET-1-induced ANF expression (32.33±3.26%) was significantly inhibited when compared to ET-1-stimulated GFP expressing cells (58.32± 4.84%, $p<0.05$). To determine whether Ca^{2+} entry arising through voltage gated channels was required for ET-1-induced hypertrophy, ET-1 was applied to cells in which voltage channels were blocked with nifedipine (10 μM) and mibefridil (1.8 μM). The percentage of cells expressing ANF was significantly lower in these inhibited cells (11.08±1.66) in comparison to controls (46.41±2.49, $p<0.05$). Under these conditions, ET-1 significantly increased the percentage of ANF expressing cells (43.46±12, $p<0.05$). Together, these data demonstrate that $InsP_3$-induced Ca^{2+} release is required for the induction of hypertrophy as a result of Gq stimulation or increased workload. Furthermore, this provides a mechanism by which gene transcription can be isolated from the increases in cytosolic Ca^{2+} that occur during every heart-beat.

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C83

NAADP-induced neuronal differentiation

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Nicotinic acid adenine dinucleotide phosphate (NAADP) is a potent NADP analogue that acts as an intracellular calcium mobilizing messenger by targeting lysosomal-like calcium stores. In this study, we compared the effects of NAADP with those of the prototypical messenger inositol trisphosphate on cytosolic calcium levels and differentiation of PC12 cells and neural stem cells. We demonstrate that liposomal delivery of NAADP mediated a robust and sustained increase in cytosolic calcium in PC12 cells and neural stem cells (isolated from olfactory bulb and subventricular zone of postnatal rat brain). The NAADP-induced calcium signal had the pharmacological characteristics of the calcium release from acidic calcium stores. This stimulus was sufficient to drive differentiation of the cells to a neuronal-like phenotype. However, in contrast, the cell fate was unaffected by more transient calcium signals generated by inositol trisphosphate-evoked release of endoplasmic reticulum calcium stores. Our data establish for the first time (i) the presence of novel NAADP-sensitive calcium stores in PC12 cells and neural stem cells, (ii) a role for NAADP in differentiation, and (iii) that calcium-dependent function can be messenger-specific. Thus, differential recruitment of intracellular calcium mobilizing messengers and their target calcium stores may represent a robust means of maintaining stimulus fidelity in the control of calcium-dependent cell function.

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SA41

Dysfunctional ryanodine receptors in cardiac disease

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The ryanodine receptor (RyR) family is a class of intracellular calcium channel that is responsible for mediating the calcium efflux from the endoplasmic reticulum (ER; or in muscle the sarcoplasmic reticulum, SR). Upon activation, the RyR produces an elevation in cytoplasmic free calcium that can trigger numerous calcium-activated physiological pathways in a variety of cells. The RyR is the largest known membrane protein and exists as three distinct isoforms (RyR1, 2, and 3) each formed by four identical subunits of approximately 5000 amino acids with a molecular mass of 560,000 Daltons, culminating in a ~2.3 megadalton complex. Electron microscopy of the purified complex has indicated the protein resembles a mushroom shape, with the stalk, or transmembrane domain, constituting 10-20% of the molecule, a region that is predicted to be at the C-terminal end. The opening and closing of the intrinsic calcium efflux pathway within the RyR is regulated by many physiological (e.g. calcium, magnesium, adenine nucleotides, pH and redox) and pharmacological (e.g. ryanodine, caffeine, tetracaine, neomycin, ruthenium red) effectors, as well as by a diverse array of cytoplasmic (e.g. FKBP12, calmodulin), lumenal (e.g. calsequestrin) and integral SR membrane (e.g. triadin, neighbouring RyRs) proteins.

The most studied physiological process involving the RyR is that of excitation-contraction coupling in striated muscle, where plasma membrane excitation is transmitted to the cell interior and results in RyR-mediated calcium efflux to trigger myocyte contraction. Mutations in the skeletal muscle RyR (RyR1) are known to be responsible for the clinical syndromes of malignant hyperthermia and central core disease, which are thought to be mediated by aberrant SR calcium release due to dysfunctional RyR1.

Recently, single residue mutations in the RyR2 have also been identified in families that exhibit catecholaminergic polymorphic ventricular tachycardia (CPVT), a condition in which physical or emotional stress can trigger severe tachyarrhythmias that can lead to sudden cardiac death. The mutations in RyR2, which currently number over 60, are distributed throughout the linear RyR2 sequence, although clustering of mutations appears to occur in the N- and C-terminal domains, as well as in a central domain of the RyR2. Further, a critical signalling role for dysfunctional RyR2 has also been implicated in the generation of arrhythmias in the common condition of heart failure.

We have prepared a cDNA expression plasmid encoding the 15 kilobase human cardiac RyR2 and have introduced various reported mutations into this construct to enable mammalian cell expression and analysis of dysfunctional calcium release mediated by the wild type and mutated RyR2. These studies suggest that the mutational locus may be important in the mechanism of calcium channel dysfunction.

Understanding the causes of aberrant calcium release via RyR2 may assist in the development of effective treatments for the ventricular arrhythmias that often leads to sudden death in heart failure and in CPVT.

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SA42

Inter-domain interactions within ryanodine receptors

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Excitation-contraction coupling in skeletal and cardiac muscles shares some basic features, and has some tissue-specific characteristics as well. The most important common feature underlying both tissues is that the ryanodine receptor (RyR) plays a central role in the activation process. In the skeletal muscle RyR (RyR1), the voltage sensing by the T-system voltage sensor activates the SR Ca^{2+} release channel by mediation of a physical interaction between the DHP receptor and the RyR1. In the cardiac muscle RyR (RyR2), the voltage-sensing process opens the T-system Ca^{2+} channel, causes Ca^{2+} flux into the cytoplasm, and this Ca^{2+} activates the RyR2. However, only a limited amount of information is available about how the excitation signal received by the RyR leads to the channel opening and contraction in normal and disease conditions. An essential step for the understanding of such a mechanism is to identify the critical regulatory domains involved. In searching for these key domains, particular attention was paid to the fact that the reported sites of point mutations in the skeletal (MH, CCD) and cardiac (CPVT and ARVD2) diseases are localized in three rather restricted regions ('hot' Regions 1-3)[1], suggesting that at least these three 'hot' domains are critical domains worth investigating.

A large amount of data accumulated by our recent work support a 'domain-switch' hypothesis [2] that involves inter-domain interactions between Region 1 and Region 2 of RyRs serving as a key mechanism for Ca^{2+} channel regulation (designated as 'domain switch'). In short, in the resting or non-activated state, Regions 1 and 2 make close contact via several sub-domains. The conformational constraints imparted by the 'zipped' configuration of these two domains stabilize the closed state of Ca^{2+} channel. Under usual stimulating conditions, the inter-domain contacts are weakened leading to an 'unzipped' or channel activating configuration. If mutation occurs in one of these domains, the inter-domain interaction will weaken even under resting conditions, causing a partial 'unzipping'; this results in a lowering of the energy barrier necessary for channel opening. Such a partially 'unzipped' domain pair is readily turned to its fully opened configuration by weaker-than-normal stimuli, causing the phenotype usually seen in the diseased states (increased sensitivity to the activation signal, incomplete closure of the channel at the resting state, etc.).

Synthetic peptides and antibodies corresponding to the domain switch cause domain unzipping and activate Ca^{2+} channels. The use of the peptides, as a structural and functional probe, permitted us to identify the sites of their interaction, monitor local conformational changes (the extent of domain unzipping) using a fluorescent probe attached in a site-directed manner, and follow the outcome of domain unzipping to the Ca^{2+} channel function. We found a close parallelism between the extent of domain unzipping and the extent of channel activation. Furthermore, dantrolene, the therapeutic agent used to treat MH, was found to reverse domain unzipping and prevents channel dysfunctions produced by the weakened inter-domain interaction. All of these findings well support the concept that the inter-domain interaction between Region 1 and Region 2 is the governing mechanism of Ca^{2+} channel regulation; aberration of this mechanism is the primary cause of RyR-linked muscle diseases, and it represents a new therapeutic target. The recent studies on the cardiac RyR2 [3] suggest that the same domain unzipping hypothesis accounts for the mechanism of the development of cardiac hypertrophy.

The remaining important question concerns Region 3, where an increasing number of RyR1 and RyR2 mutations have been reported in recent years, but whose role has not yet been thoroughly investigated. According to the recent report [4], the 3722-4610 region of RyR2, designated as I-domain, may be involved in specific inter-domain interactions. Our preliminary studies have shown that the peptide probe matching the C-terminal area of Resion 3 binds to the N-terminal portion of Region-3 and modulates the channel gating in a Ca^{2+}-dependent manner, consistent with the view that the interaction between the N-terminal portion of Region 3 and the channel pore region is involved in channel gating.

Abbreviations: ARVD2, arrhythmogenic right ventricular dysplasia type-2; CCD, central core disease; CPVT, catecholaminergic polymorphic ventricular tachycardia; DHP, dihydropyridine; MH, malignant hyperthermia.

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SA43

Regulation of inositol 1,4,5 trisphosphate (IP_3) receptors and IP_3-induced Ca^{2+} release

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IP_3 receptors are ligand-operated intracellular Ca^{2+} channels, which are involved in the generation of cellular Ca^{2+} signals in many different physiological and pathological conditions. Control of IP_3-induced Ca^{2+} release (IICR) is possible either by regulation of the ligand binding, thus affecting the affinity for IP_3, or by changing the coupling between ligand binding and channel opening. The latter mechanism is much less understood but many data indicate that malfunction of this coupling mechanism may either produce an inactive channel or inversely may produce an hyperactive or even a leaky channel (Szlufcik et al. 2006). A functional element, which is involved in both types of regulation is the 'suppressor/coupling' domain; this is the N-terminal 225 AA of the IP_3R. This suppressor/coupling domain attenuates the interaction between IP_3 and the IP_3-binding core, but it is also essential for activation of IICR. By using chimeric IP_3Rs, in which the suppressor domains of IP_3R1 and IP_3R3 were exchanged, we have shown that the suppressor was an important determinant of the affinity of these different isoforms. Moreover, deletion of 11 AA in IP_3R1 eliminated the suppression effect and yielded a high affinity [Δ76-86]-IP_3R1 mutant.

Among the proteins that directly control IICR, we have particularly focused on calmodulin (CaM). We have demonstrated that CaM and CaM-like proteins such as CaBP1 shared a Ca^{2+}-independent binding site on the suppressor domain (AA 49-81) in a position which is critical for determining the IP_3 affinity. We showed that CaM did not function as a Ca^{2+} sensor, though inhibition of IICR by CaM only occurred in the presence of Ca^{2+}. CaM also binds to a less conserved Ca^{2+}-dependent binding site in middle portion of IP_3R1, but no regulatory role on IICR was as yet demonstrated. In addition we have found that endogenously bound CaM is essential for IICR. Depletion of CaM by high-affinity CaM-binding peptides reversibly inhibited IICR. This effect was on the coupling function as IP_3 binding was not affected. The data suggested that CaM is constitutively associated with the IP_3R and is essential for proper coupling between IP_3 binding and channel activation.

There is increasing evidence that IP_3Rs play an essential role in apoptosis and that multiple mechanisms may be involved (Hanson et al. 2004). We observed that increasing the affinity for IP_3 in the [Δ76-86]-IP_3R1 mutant directly increased staurosporin (STS)–induced apoptosis. In addition however IP_3R1 is a substrate for caspase-3 cleavage, which yields a C-terminal 95kDa fragment containing the domains responsible for tetramerisation and for formation of a constitutively active channel. We have demonstrated that caspase-3 cleavage occurred during STS-induced apoptosis and that it may represent a positive feedback mechanism accelerating cell death (Assefa et al. 2004). Although the caspase-3 site is only present in IP_3R1 we have evidence that a similar truncation may occur for IP_3R3 as a consequence of calpain activity.

Truncation of IP_3R1 by caspase-3 is an extreme case illustrating the possibility that IP_3Rs may operate as IP_3-independent or leaky channels. Such behavior was also observed as a consequence of IP_3R1 hyper-phosphorylation (Oakes et al. 2005). The Ca^{2+} leak may affect the luminal Ca^{2+} content which is a critical parameter controlling cell death pathways. It is conceivable that such leak pathways may occur particularly in some pathological conditions such as Alzheimer's disease. We have observed a specific, five-fold up-regulation of IP_3R1 in mouse embryonic fibroblast deficient in presenilin-1 and -2 (MEF_{DKO}). MEF_{DKO} cells showed a decrease in the $[Ca^{2+}]_{ER}$ as measured by ER-targeted aequorin luminescence and the lower $[Ca^{2+}]_{ER}$ was associated with an increase in a Ca^{2+} leak from the ER. Using RNA-interference-mediated reduction of IP_3R1 we could demonstrate that the up-regulation of this isoform was responsible for the

increased Ca^{2+} leak and the lowered $[Ca^{2+}]_{ER}$ in PS_{DKO} cells. We also showed that the decreased $[Ca^{2+}]_{ER}$ in PS_{DKO} cells was protective against apoptosis.

In conclusion, our data indicate that IP_3Rs may be directly regulated at the level of the apparent affinity for IP_3. In addition, parameters that affect the structural requirements for proper channel opening may result in inactivation or in the formation of hyperactive channels or even in the generation of an IP_3-independent Ca^{2+} leak. Whereas an increased Ca^{2+} mobilization can contribute to pro-apoptotic mechanisms, Ca^{2+} leak pathways may also affect the Ca^{2+} store content. Such a decrease of the ER Ca^{2+} content may then be operative as an anti-apoptotic mechanism.

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SA44

Dissecting IP_3 receptor function in *C. elegans*

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Signalling through the second messenger IP_3 and its receptor (IP_3R) is a central mechanism by which extracellular signals regulate intracellular calcium signals. In order to dissect the functions of IP_3 signalling in whole animals we have been using the nematode *Caenorhabditis elegans*. IP_3Rs in *C. elegans* are encoded by a single gene, *itr-1*. Using a combination of genetic, RNAi and transgenic dominant-negative approaches the functions of the IP_3 signalling pathway in *C. elegans* are being identified. Results from this work reveal that IP_3 signalling is widely involved in non-neuronal ultradian rhythmic processes; in particular feeding, defecation and ovulation (see 1 and references therein). IP_3 signalling is also involved in developmental processes. Embryos with disrupted IP_3 signalling have defects in differentiation and in morphogenesis (2,3). Thus IP_3Rs are involved in a wide range of processes in *C. elegans*.

The ability of IP_3Rs to function in such a diverse range of activities is likely to require complex differential regulation. Such regulation may stem from the upstream and downstream components of the particular IP_3-mediated pathway used in a process and also from regulation by interaction with other proteins and ligands. A substantial number of potential and putative regulatory interactions have been identified in IP_3Rs from a range of sources. These include interactions with other proteins and with small ligands such as ATP. Although much of this work has been performed with mammalian IP_3Rs the binding sites for some of these interactors are also found in the *C. elegans* receptor. We have recently established a system which allows us to modify the IP_3R genomic DNA using homologous recombination in *E. coli* and the reintroduce these modified *itr-1* genes into *C. elegans*, in particular into *C. elegans* with reduced or IP_3R function. We are then able to test the effects of modifying particular binding sites on the ability of IP_3Rs to rescues particular functions. We have used this system to test the importance of two sites (a) a putative ATP binding site and (b) the binding site for FKBP12. Disruption of the ATP binding site appears to have modest effects on *itr-1* function whereas disruption of the FKBP12 sites appears to have severe effects on function. This system allows us to test the importance of regulatory sites, identified using biochemical and molecular approaches, in whole animals.

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