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Regulation of cell growth and cell death by MAP kinase signalling pathways

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The role of $InsP_3$ signalling cascade in the generation of cardiac arrhythmia

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Signalling networks regulate apoptosis during inner ear development

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Programmed cell death is a critical process for normal development and tissue homeostasis. While the basic programme of execution of apoptosis is conserved, distinct regulatory signals have been described depending on the cell type and developmental stage. Particularly interesting are the opposite actions displayed by nerve growth factor (NGF), which acts either as a survival factor or as a death-inducing factor. A coherent understanding of the regulation of programmed cell death during development requires a co-ordinated study of some of the multiple signals acting on the cells. We are interested in the molecular mechanisms by which these signals initiate and pattern the vertebrate inner ear. IGF-1 is a member of a family of structurally related genes that have pleiotropic actions on embryonic cells. *In vitro* culturing and knock-out analysis with mice have determined that IGF-1 is critical for the proper development and maturation of the inner ear. IGF-1 stimulates the generation of lipidic second messengers, activates the Raf/mitogen-activated protein kinases cascade and increases AP-1 and PCNA levels leading to cell growth and survival. In contrast, NGF, after binding to p75 low affinity receptors, increases ceramide levels and activates Jun N-terminal kinase and caspase-3, in a process that regulates apoptotic cell death. In this context, we have explored the interactions between the pathways activated by IGF-1 to prevent apoptosis and those activated by NGF to induce cell death. We propose that the dynamic balance between levels of ceramide metabolites and the consequent regulation of Akt phosphorylation are important factors that determine whether a cell survives or dies.

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S30

Aequorin imaging of subcellular calcium dynamics in excitable cells

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Intracellular calcium regulates many cell functions including contraction, secretion, respiration, etc. These functions are carried out inside discrete organelles (nucleus, mitochondria, ...) or functional domains (subplasmalema, caveolae, ...). As a consequence, studies on calcium signalling are evolving from the cellular to the subcellular environment. Classical calcium probes are difficult to target to organelles and often lack the proper affinity for calcium. The cloning of aequorin and determination of its structure enabled its targeting and fitting of its calcium affinity to monitor subcellular calcium signals. In addition, the use of ultrasensitive photon-counting photomultipliers (aequorimeters) or photon-counting cameras allows also recording of subcellular signals from individual cells. We have used targeted aequorins to monitor nuclear, cytosolic and mitochondrial calcium dynamics in single cells (Villalobos *et al.* 2001). Clonal GH3 cells and normal mouse anterior pituitary cells were infected with herpes virus delivering either nuclear, cytosolic or mitochondrial aequorins. After 24–48 h, cells were incubated for 1–2 h with coelenterazines to reconstitute the active enzyme. Then, cells were subjected to photon-counting imaging. With this methodology, we recorded spontaneous oscillations of calcium concentration in all three compartments. It is well established that pituitary cells display spontaneous cytosolic calcium oscillations secondary to electric activity. Calcium oscillations in all three compartments were inhibited by external calcium removal, blocking of calcium channels or plasma membrane hyperpolarization. Oscillations were, however, not affected by emptying of the intracellular calcium stores with thapsigargin. Thryrotropin releasing hormone (TRH), a hypophysiotrophic factor that enhances electric activity, increased oscillations in all three compartments. However, the frequency and/or amplitude of spontaneous calcium oscillations and its stimulation by TRH recorded in the cytosol markedly differed from those recorded in the nucleus or mitochondria. Specifically, whereas the signals in the nucleus were clearly dampened with respect to those in the cytosol, oscillations in the mitochondria were clearly enlarged severalfold. Thus, the same mechanism, namely electric activity, generates different patterns of calcium oscillations in the cytosol, the nucleus and mitochondria. In addition, we found evidence indicating that oscillations occur only in those mitochondria functionally coupled to plasma membrane calcium channels and sensing subplasmalema high calcium concentrations. The remaining mitochondria barely sensed nor underwent oscillations. Since calcium is known to regulate several mitochondrial dehydrogenases, our results indicate that spontaneous mitochondrial calcium oscillations regulate basal ATP synthesis. In support of this view, we found that abolition of oscillations by external calcium removal or calcium channel blockade decreased resting intracellular NAD(P)H levels. Moreover, since oscillations are only generated in a subpopulation of mitochondria, then we conclude that control of ATP synthesis is driven by electric activity in that population but not in the remaining mitochondria (Villalobos *et al.* 2001).

Regarding the nucleus, the dampening of nuclear calcium oscillations relative to those recorded in the core cytosol can be explained by either a permeability barrier at the nuclear envelope or, alternatively, by an increased buffer capacity of the nucleus (P. Chamero, C. Villalobos, M.T. Alonso & J. García-Sancho, submitted). At present, we do not have evidence to favour either of the two alternatives. However, whatever the mechanism, the functional consequence is that transmission of the cytosolic calcium signal to the nucleus depends on the signal's naturel. Specifically, sustained calcium increases in the cytosol are faithfully transmitted into the nucleus whereas transient, oscillatory signals are strongly dampened when sensed by the nucleus. This may be a mechanism by which calcium may regulate differentially nuclear functions such as cell division or gene expression (P. Chamero, C. Villalobos, M.T. Alonso & J. García-Sancho, submitted).

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S31

Voltage control of P2Y receptor-evoked Ca^{2+} release in the rat megakaryocyte: sensitivity limits and activation by action potentials

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The ability of membrane potential to modulate IP_3-dependent Ca^{2+} release independently of Ca^{2+} influx has been described in a number of excitable cell types but has been most extensively studied in the non-excitable rat megakaryocyte (MK) (Mahaut-Smith *et al.* 1999). We have now examined the limits of sensitivity of this phenomenon to step depolarisations and activation by action potential waveforms (APW) in the MK.

Male Wistar rats were humanely killed by CO_2 inhalation and cervical dislocation. Whole-cell patch clamp and $[Ca^{2+}]_i$ recordings from marrow MKs were conducted as described previously (Mahaut-Smith *et al.* 1999). Depolarising steps of increasing magnitude or duration were applied during activation of P2Y receptors (1 μM ADP). The peak $[Ca^{2+}]_i$ response showed marked heterogeneity between cells: range 23–560 nM ($n = 17$) for a 55 mV, 5 s step from −75 mV. A positive correlation was observed between the average $[Ca^{2+}]_i$ increase and amplitude of the voltage step (range 3 to 55 mV) from −75 mV. The responses for 55, 20 and 10 mV steps were 151 ± 34, 84 ± 13 and 45 ± 6 nM, respectively (mean ± S.E.M. $n = 17$ or 18). The most sensitive cells lacked a threshold potential and showed $[Ca^{2+}]_i$ increases with depolarisations of only 3 mV ($n = 5$). We also examined the dependence of the depolarisation-evoked $[Ca^{2+}]_i$ increase upon pulse duration using a step from −85 to +50 mV. The $[Ca^{2+}]_i$ response increased with pulse duration up to 700 ms; the average was 48 ± 15 nM ($n = 9$) for 250 ms compared to 25 ± 4 nM, $n = 17$ for 100 ms. Again, the most sensitive cells responded with a $[Ca^{2+}]_i$ increase to the shortest (25 ms) pulse.

These data suggest that depolarisations of only a few millivolts or tens of milliseconds are sufficient to induce a $[Ca^{2+}]_i$ increase via modulation of P2Y receptor-induced Ca^{2+} release. This implies that a variety of voltage waveforms including action potentials can cause Ca^{2+} release via this mechanism. To further test this concept, atrial and ventricular cardiac APWs were applied during stimulation of MKs with 1 μM ADP. These APWs were both capable of inducing a $[Ca^{2+}]_i$ increase, but the ventricular waveform generated a larger peak response (182 ± 12 nM, $n = 6$ *vs.* 47 ± 6 nM, $n = 10$).

Our data indicate that voltage control of Ca^{2+} release via a G-protein-coupled receptor is sufficiently sensitive to detect small voltage changes and APW. The role of this novel signalling mechanism in excitable and non-excitable tissues should therefore be considered.

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