Calcium signalling and synaptic plasticity at single hippocampal synapses

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Analysis of synaptic transmission in the brain using purely electrophysiological techniques has proved problematic because of the large number of synapses on most neurons. The advent of confocal microscopy has made it possible to circumvent this problem by studying synaptically evoked calcium transients at single dendritic spines in organised brain tissue. We have examined two kinds of synapse in organotypic hippocampal cultures prepared from 8 day postnatal male Wistar rats, maintained for 14–21 days *in vitro* prior to recording: associational and Schaffer-collateral synapses at small spines on distal apical dendrites of CA3 and CA1 pyramidal cells, respectively, and mossy fibre synapses at complex dendritic spines (Cajal's 'thorny excrescences') on proximal dendrites of CA3 pyramidal cells. Cells were filled with a high-affinity Ca^{2+} indicator (Oregon Green 488 BAPTA-1, Molecular Probes), and activated by stimuli to stratum radiatum (to activate Schaffer/associational fibres), or to granule cells (to activate mossy fibres). At Schaffer/associational synapses, the synaptically evoked calcium transient is NMDA-receptor dependent, and is abolished by drugs that suppress release of calcium from intracellular stores (Emptage *et al.* 1999). Consistent with this finding, in serial-section EM studies using immunogold labelling of ryanodine receptors (RyRs), we find that RyRs are expressed in over 60 % of spines in distal apical dendrites of CA1 pyramidal cells. A slightly lower proportion of spines displayed immuno-reactivity for the RyR accessory protein, sorcin. In contrast, the main source of the synaptically evoked calcium transient in thorny excrescences is calcium entry through NMDA receptors and voltage-dependent calcium channels (Reid *et al.* 2001).

We have used synaptically evoked postsynaptic Ca^{2+} transients to analyse changes in the quantal components of synaptic transmission at individual Schaffer/associational and mossy fibre synapses following the induction of long-term potentiation (LTP). In 8/13 Schaffer/associational synapses, LTP was accompanied by an increase in the probability of a calcium transient (P_{Ca}) 30 min post-induction (mean pre-tetanus *vs.* post-tetanus values of P_{Ca}, ± S.E.M., for the group of 13 spines was 0.26 ± 0.07 pre-LTP *vs.* 0.56 ± 0.10 post-LTP, $P < 0.007$, 2-tailed paired *t* test); in a control population of 14 spines, imaged on cells in which LTP was blocked by D-AP5 (50 μM), or in which low-frequency trains were delivered that failed to induce persistent changes in the intracellularly recorded EPSP, there was no change in P_{Ca} over a similar time period (P_{Ca} was 0.33 ± 0.06 pre-treatment *vs.* 0.35 ± 0.06 post-treatment, n.s). An increase in the amplitude of the calcium transient was also observed in many cases 30 min after the induction of LTP. These observations indicate a presynaptic component to the early phase of LTP at Schaffer/associational synapses, as well as a novel postsynaptic biochemical component of LTP; they do not preclude additional postsynaptic components of LTP expression. At mossy fibre synapses, where expression of LTP is believed to be exclusively presynaptic, we found that an increase in P_{Ca} was also common; in some cases the increase in P_{Ca} was accompanied by the emergence of higher amplitude synaptically evoked calcium transients which at these multi-release site synapses can be explained by the recruitment of additional release sites following the induction of LTP. In some cases, mossy fibre LTP was associated with the activation of previously silent synapses.

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We thank Dr F.A. Lai for pan-RyR antiserum, and Dr M. Meyer for sorcin antiserum. This work was supported by the MRC, and grants from the Human Frontier Science Program, European Union Framework 5, and Canadian Institutes of Health Research. C.A.R. was a Howard Florey Fellow of the Royal Society; R.F.-F. is a Killam Fellow (Canada).

All procedures accord with current local guidelines.

Prostaglandins mediate a major component of astrocyte calcium elevations responsible for receptor-stimulated glutamate release

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It has been recently demonstrated that glial cells are active participants of synaptic transmission and brain circuitry. In particular, astrocytes are active players of rapid chemical communication in the brain, but their signalling modes remain largely undefined. Here we show that stimulation of two different receptors for neurotransmitters (mGluR5 for glutamate and B2 for bradykinin) in cultured astrocytes, activates a Ca^{2+}-dependent glutamate release which in all cases is sensitive to inhibitors of exocytosis such as tetanus neurotoxin and bafilomycin A1 (−85 % with either agent) suggesting that they share a common mechanism of action which may occur via a mechanism resembling neuronal vesicular release. A surprising feature of this releasing cascade is the inhibition of the glutamate released in the presence of cyclo-oxygenase (COX) blockers such as aspirin and indomethacin (−70/80 % with either agent). The role of the prostaglandins (PGs) in this mechanism is not fully understood, but we have found with Ca^{2+} imaging studies at the single-cell level that receptor-stimulated $[Ca^{2+}]_i$ elevations in astrocytes are dramatically reduced (on average, −60/70 % of the AUC) in about 75 % of cells and completely abolished in the others, in the presence of COX inhibitors. Moreover, receptor stimulation is accompanied by PGE_2 formation and its release in extracellular medium, suggesting that PGs are implicated in the amplification of the Ca^{2+} signalling and cell-to-cell communication in the astrocytic network. Finally, administration of exogenous PGE_2 produced *per se* rapid, store-dependent, Ca^{2+} responses. All these data indicate that : (a) COX-dependent $[Ca^{2+}]_i$ elevations are necessary for glutamate release; (b) COX products are responsible for a major component of astrocyte $[Ca^{2+}]_i$ elevations in response to glutamate or bradykinin; (c) a large part (albeit not all) of such a component is sustained by the autocrine/paracrine action of PGE_2.

All procedures accord with current National guidelines.

Neuronal calcium homeostasis and ageing

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Numerous studies have found altered Ca^{2+} homeostasis in neurons during the ageing process. However, it remains unclear whether there is one primary initiating dysfunction or whether multiple alterations develop in parallel. Previously, we found that the density of available L-type Ca^{2+} channels increases in rat hippocampal neurons during ageing (Thibault & Landfield, 1996). Recently, we found that during repetitive (7 Hz) synaptic stimulation in hippocampal slices, $[Ca^{2+}]$ rises more in neurons from aged than from young adult rats. However, this difference becomes apparent only after several seconds of stimulation above spike threshold. The elevated $[Ca^{2+}]$ is correlated with impaired short-term synaptic frequency facilitation, a correlate of deficient memory. Facilitation was also impaired by exposure to Bay K 8644, an L-type channel agonist (Thibault *et al.* 2001). The delayed $[Ca^{2+}]$ elevation in aged neurons is potentially consistent with several possible mechanisms of Ca^{2+} dyshomeostasis, including greater influx, impaired sequestration or extrusion and/or impaired mitochondrial function. However, recent findings in a hippocampal culture model suggest that sustained Ca^{2+}-induced Ca^{2+} release can be triggered by elevated Ca^{2+} influx (Clodfelter *et al.* 2002) and therefore, also could account in part for the delayed $[Ca^{2+}]$ elevation in aged neurons.

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All procedures accord with current local guidelines.

Intra-ER calcium dynamics in nerve cells

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The endoplasmic reticulum (ER) is an intracellular organelle of fundamental importance present in all types of eukaryotic cells. The ER lumen is densely packed with numerous enzymatic systems that allow protein synthesis in the rough endoplasmic reticulum and correct post-translational 'folding' of these proteins. Any malfunctions in the latter process result in accumulation of unfolded proteins, which in turn activates several signalling systems aimed at appropriate compensatory responses. At the same time the ER is recognised as an important component of a different signalling system, i.e. the cytosolic calcium signalling cascade. Within a framework of this cascade the endoplasmic reticulum serves as a rapidly exchanging Ca^{2+} store, able to release Ca^{2+} ions upon appropriate physiological stimulation. In order for the endoplasmic reticulum to work as a dynamic Ca^{2+} store, a high concentration of free Ca^{2+} has to be maintained within its lumen, where $[Ca^{2+}]$ varies between 0.2 and 1 mM. Simultaneously, high intraluminal free Ca^{2+} concentration appears to be a key factor determining the activity of synthesis and processing of proteins within the endoplasmic reticulum, and disruption of endoplasmic reticulum Ca^{2+} homeostasis triggers endoplasmic reticulum stress response.

The importance of the endoplasmic reticulum as a dynamic calcium pool in nerve cells was first appreciated in the late 1980s and early 1990s when several groups reported stimulation-induced cytosolic Ca^{2+} signals recorded from cultured neurones, which were not affected by Ca^{2+} removal from the extracellular media. These $[Ca^{2+}]_i$ signals were triggered by either caffeine (potent activator of RyRs) or by neurotransmitters (glutamate or ATP) stimulating metabotropic (i.e. $InsP_3$ producing) receptors (Verkhratsky & Shmigol, 1996; Verkhratsky & Petersen, 1998).

By imaging free Ca^{2+} concentration within the ER lumen ($[Ca^{2+}]_L$) of cultured peripheral (dorsal rot ganglia) and central (hippocampus) neurones employing low-affinity Ca^{2+} dyes we succeeded in real-time visualisation of $[Ca^{2+}]_L$ dynamics in response to physiological and pharmacological stimulation.

In a study of cultured rat DRG neurones we deployed the patch-clamp whole-cell technique to record membrane currents in parallel with simultaneous video-imaging of both $[Ca^{2+}]_L$ and calcium concentration in cytosol ($[Ca^{2+}]_i$) using low (Mag-Fura-2) and high (Fluo-3) affinity Ca^{2+}-sensitive fluorescent probes (Solovyova *et al.* 2002). Activation of ryanodine receptors by caffeine triggered a rapid fall in $[Ca^{2+}]_L$ levels to 40–50 % of the resting $[Ca^{2+}]_L$ value. Ca^{2+} entry through voltage-gated calcium channels also induced a transient decrease in $[Ca^{2+}]_L$. This $[Ca^{2+}]_L$ response was inhibited by 50 μM ryanodine and potentiated by 1 mM of caffeine, indicating that it was directly associated with calcium-induced calcium release (CICR) triggered. Surprisingly the amplitude of CICR was linearly dependent on the amount of Ca^{2+} ions entering the cell with the Ca^{2+} current.

The $InsP_3$-induced Ca^{2+} release was investigated in saponin-permeabilised DRG neurones pre-loaded with Mag-Fura-2. The permeabilisation effectively removed the sytosolic portion of the dye allowing direct $[Ca^{2+}]_L$ monitoring in response to $InsP_3$ application. We found that both Ca^{2+} release mechanisms, the RyR-mediated and the $InsP_3$R-mediated, share the same Ca^{2+} pool, further substantiating the idea of one continuous ER Ca^{2+} store (Park *et al.* 2000).

In central neurones the visualisation of Ca^{2+} was hampered by the prolonged process of removal of the excess of low-affinity Ca^{2+} indicator from the cytosol. By using non-invasive staining with single-wavelength low-affinity Ca^{2+} indicators Mag-Fluo-4 (K_d ~20 μM) and Fluo-3FF (K_d ~40 μM) in combination with confocal imaging we found high levels of resting fluorescence within the cytosol, whereas virtually no signal was detected in the nuclear region. Cell depolarisation triggered a rapid transient increase in fluorescence in the nucleus. This 'nuclear' response was greatly attenuated by cell incubation with 100–500 nM thapsigargin. Thus inhibition of Ca^{2+} accumulation into the ER prevented the spread of Ca^{2+} elevation into the central portions of the cell. We suggest that the ER lumen may represent a specific Ca^{2+} 'tunnel' (similar to that found in pancreatic acinar cells – Petersen *et al.* 2001) which provides a pathway for rapid Ca^{2+} transport within the cell, thus connecting plasma membrane with cell interior.

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This research was supported by a BBSRC research grant (ref. 34/C12751).

All procedures accord with current local guidelines.

Mitochondrial Ca^{2+} homeostasis in cell life and death

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Functional activity of mitochondrial networks

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Roles of mitochondria, calcium and NO in glutamate excitotoxicity

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Although it has been known for many years that isolated respiring mitochondria have a massive capacity to accumulate calcium, the physiological significance of this pathway has remained uncertain until very recently. In the last few years, it has become clear that, in most cells, physiological Ca^{2+} signals are associated with significant mitochondrial Ca^{2+} uptake. Under pathological conditions, mitochondrial Ca^{2+} uptake may be a crucial step in the progression to cell death. During episodes of hypoxia in the CNS, the excitatory transmitter glutamate accumulates in the extracellular space. Prolonged stimulation of glutamate (and particularly NMDA) receptors is toxic to neurons, mediated largely by an increase in intracellular calcium concentration ($[Ca^{2+}]_c$). We have made simultaneous measurements of mitochondrial potential ($\Delta\psi_m$) and intracellular $[Ca^{2+}]_c$ in rat hippocampal neurons in culture. Application of glutamate sufficient to cause 70–80 % cell death was associated with the collapse of $\Delta\psi_m$. In contrast, raising $[Ca^{2+}]_c$ simply through depolarisation with 50 mM KCl had no significant impact on $\Delta\psi_m$, even when the $[Ca^{2+}]_c$ levels achieved were similar to those seen with glutamate. Examination of the glutamate responses cell by cell showed a highly heterogeneous mitochondrial response, both in time course and amplitude while the $[Ca^{2+}]_c$ responses were highly homogeneous. Thus, the mitochondrial responses did not show a simple quantitative relationship to the change in $[Ca^{2+}]_c$. We therefore sought other variables that might contribute to the response. Obvious candidates were superoxide and NO. No increase in superoxide production (measured with hyodroethidine fluorescence) was detectable in response to glutamate and the mitochondrial response to glutamate was not altered by a range of antioxidant scavengers (Vergun *et al.* 2001). Notably, a combination of antioxidants inhibited the glutamate current and suppressed the glutamate-induced rise in $[Ca^{2+}]_c$, suggesting that great care must be used in interpreting data using such reagents. Inhibition of nNOS suppressed the change in $\Delta\psi_m$ (Keelan *et al.* 1999) without altering the $[Ca^{2+}]_c$ response. Further, in the presence of an NO donor, the $[Ca^{2+}]_c$ response to 50 mM KCl now caused a mitochondrial depolarisation. Thus it seems that the collapse of $\Delta\psi_m$ with glutamate requires the synergistic action of both NO and Ca^{2+}. The 'source specificity' of glutamate-mediated increase in $[Ca^{2+}]_c$ compared with that mediated by KCl is probably mediated by the localisation of nNOS close to NMDA receptors by the scaffolding protein PSD-85 (Sattler *et al.* 1999) so that domains of $[Ca^{2+}]_c$ sufficient to activate nNOS only occur close to NMDA receptors and not close to voltage-gated Ca^{2+} channels. The other major issue to consider is the mechanism underlying the collapse of mitochondrial potential. One likely candidate is the opening of the mitochondrial permeability transition pore (mPTP), a channel which opens in the mitochondrial inner membrane under pathological conditions of high $[Ca^{2+}]_m$, oxidative stress, high $[P_i]$ and ATP depletion. mPTP opening is blocked by cyclosporin A (CsA) which inhibited the glutamate-induced collapse of mitochondrial potential. However, CsA also inhibits calcineurin, so inhibiting NO production, and the mitochondrial depolarisation was also suppressed by FK506, which inhibits calcineurin but does not affect the mPTP. It therefore remains difficult to attribute the glutamate-induced loss of mitochondrial potential unequivocally to mPTP opening without more precise experimental tools.

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Regulated exocytosis: not one, but multiple, Ca^{2+}-dependent processes in neurosecretory cells

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Regulated exocytosis is known as the operational mechanism by which specific organelles, secretion granules/vesicles, fuse their membrane with the plasmalemma in response to an appropriate signal and thus release their content to the extracellular space. Other forms of exocytosis exist, concerning for example vesicles generated at the trans-Golgi network and recycling endosomes. In the latter case, however, membrane fusion is not regulated but constitutive. The two processes are therefore believed to be substantially different, both in terms of mechanisms and physiological significance. Based on this (by the way, almost generally accepted) view, regulated exocytosis is the process responsible for quantal secretion and exists therefore in specialized secretory cells (neurons, endocrine, exocrine, etc.) while the other cells are competent only for constitutive exocytosis.

A few years ago, capacitance studies carried out first in fibroblasts, (i.e. non-secretory cells CHO and 3T3) then in a defective clone of the pheochomocytoma line, PC12, that our previous work had shown to be completely incompetent for regulated neurosecretion, demonstrated that $[Ca^{2+}]_i$ increases induced by photolysis of caged Ca^{2+} are able to induce a rapid increase of the cell surface, apparently similar to the response triggered by the same treatment in cells competent for neurosecretion. Detailed analysis of this response demonstrated it to be sustained by the fusion of small vesicles (< 100 nm in diameter) and to occur by a membrane fusion type independent of the classical SNAREs, syntaxin 1, VAMP 2 and SNAP 25. Working in the defective PC12 clone as well as in a variety of other cell types we have now identified the new endocytotic system. The specific organelle is a vesicle recognized by the expression of a protein we have named Marker of Exocytotic Vesicles (MarEx). This marker is localized within the vesicle and appears at the surface only by Ca^{2+}-triggered exocytosis. MarEx is expressed by many, although not all types of cells. In some of them, for example in wild-type PC12 treated with NGF,

expression occurs only during differentiation. Moreover, MarEx appears at the surface of cells which had been wounded, suggesting its participation in wound healing.

We conclude that another form of exocytosis exists in addition to the classical secretory form. In nerve cells the latter is specific for clear vesicles and dense granules. The new form is in contrast specific for another type of vesicle which might have nothing to do with secretion but be responsible for rapid enlargements of the plasma membrane. In view of this possibility we suggest the vesicles competent for this form of exocytosis to be called enlargesomes.

Calcium channel gating modulation: a manifold system to control calcium entry in neurosecretory cells

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Voltage-gated Ca channels are crucial for controlling fast Ca signalling during neurosecretion. In the chromaffin cells of adrenal medulla, the autocrine action of neurotransmitters and the production of diffusable second messengers, such as nitric oxide (NO), can induce drastic changes to Ca channel gating and to the Ca-dependent events controlling catecholamine secretion and cell activity. A functionally interesting Ca signalling pathway in these cells is the G_i/G_o protein-dependent inhibition of N- and P/Q-channels, which is activated by the neurotransmitters released during exocytosis (ATP and opioids). This feedback modulation reveals peculiar features at the single channel level (delayed activation at low voltages and fast activation after strong prepulses), which can be used as fingerprint to detect local secretion in membrane patches where Ca channels, G_i/G_o proteins and membrane autoreceptors are closely packed (Carabelli *et al.* 1998). Alternative Ca signalling pathways involve the multiple modulation of L-channels which can either up- or downregulate the gating activity of the channel. Down-modulation of L-channels can be induced either by G_i/G_o proteins activated by membrane autoreceptors (purinergic, opioidergic and adrenergic) (Carabelli *et al.* 2001) or by elevations of cGMP associated to the production of NO and the activation of PKG (Carabelli *et al.* 2002). The two mechanisms act in parallel and cause a marked reduction of L-channel gating activity, mainly by causing prolonged shut times, with no changes to mean open times, the latency to first channel opening and single channel conductance. At variance with the down-modulation of N- and P/Q-channels, both actions are voltage independent and do not cause substantial changes to the activation-inactivation kinetics of voltage-gated Ca currents.

L-channel gating can also be upregulated by autocrine pathways, mediated by cAMP/PKA and capable of increasing the probability of channel openings by enhancing the mean open time and lowering the mean shut times (Carabelli *et al.* 2001). Preliminary experiments in rat chromaffin cells indicate that Ca currents are under the control of β_1- and β_2-adrenoreceptors (β_1-ARs, β_2-ARs) stimulation. The cAMP/PKA-mediated upregulation of L-channels is primarily mediated by β_1-ARs (Carbone *et al.* 2001), through a remote mechanism of action, while the β_2-ARs are mainly coupled to PTX-sensitive G_i/G_o proteins and produce only localised inhibition.

Given that all these Ca signalling pathways are primarily autocrine (including NO production) and that the packed array of chromaffin cells in the adrenal medulla favours the accumulation of released material, it is evident that the distinct forms of Ca channel modulation described here widen the dynamic range by which chromaffin cells control their secretory activity at rest or during maximal frequency stimulation.

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All procedures accord with current National guidelines.

ER calcium movements

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The endoplasmic reticulum as a functional Ca^{2+} tunnel system in pancreatic acinar cells

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In pancreatic acinar cells, the physiological cytosolic Ca^{2+} signals consist mostly of repetitive local Ca^{2+} spikes confined to the apical granular region. These signals can be induced by physiological concentrations of acetylcholine (ACh) or cholecystokinin (CCK). The Ca^{2+} spikes are due to short-lasting openings of Ca^{2+} release channels in apical extensions of the endoplasmic reticulum (ER), which project into the granular region (Petersen *et al.* 2001). The Ca^{2+} released into the cytosol of the granular pole mostly stays in the apical area due to a mitochondrial firewall surrounding the granular region (Tinel *et al.* 1999). The local apical Ca^{2+} spikes elicit not only exocytosis, but also fluid secretion through opening of Ca^{2+}-activated Cl^- channels localized specifically in the apical plasma membrane (Park *et al.* 2001). During physiological CCK stimulation, some of the local Ca^{2+} spikes trigger global Ca^{2+} waves. Global Ca^{2+} waves are generated by a complex interplay between at least three separate intracellular Ca^{2+} release channels controlled by inositol trisphosphate, cyclic ADP-ribose and nicotinic acid adenine dinucleotide phosphate (Cancela *et al.* 2002) and depend on the process of Ca^{2+}-induced Ca^{2+} release (Solovyova *et al.* 2002).

The resting free Ca^{2+} concentration in the ER store is about 100–300 μM and depends on the balance between the passive leak and the resting active uptake mediated by the Ca^{2+} pump. The main determinant of the rate of ER Ca^{2+} pumping is the Ca^{2+} concentration in the ER lumen (Petersen *et al.* 2001; Solovyova *et al.* 2002). Experiments with local Ca^{2+} uncaging in the ER lumen have shown directly the ability of Ca^{2+} to move quickly inside the ER over considerable distances (~10 μm) (Petersen *et al.* 2001). Ca^{2+} released from the ER terminals in the granular pole can therefore quickly be replenished by Ca^{2+} drawn from the bulk of the ER store in the basal region.

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All procedures accord with current UK legislation.

Molecular physiology of ryanodine receptor type 3

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The family of ryanodine receptor (RyR) genes encodes three highly related Ca^{2+} release channels: RyR1, RyR2 and RyR3. Until about 10 years ago, RyRs were essentially known for being the Ca^{2+} release channels of the sarcoplasmic reticulum of striated muscles because of the high levels of expression of the RyR1 and RyR2 isoforms in skeletal and cardiac muscles, respectively. In contrast with the above picture, the RyR3 gene has not been found to be preferentially expressed in one specific tissue, but rather to be broadly expressed in different cell types. This broad expression pattern has been subsequently observed also for the RyR1 and RyR2 genes, which in addition to their preferential expression in striated muscles, have been found expressed also in several other cell types. An updated picture reveals therefore that in several cells of vertebrates two or even three RyR isoforms can be co-expressed. This notion has been substantiated by experiments, at a functional level, that suggest that co-expression of different RyR channel isoforms may affect specific aspects of intracellular Ca^{2+} signals and hence modulate the regulation of specific cellular functions.

Further evidence of the biochemical complexity of the mechanisms underlying the process of Ca^{2+} release stems from studies indicating that this process depends, in addition to Ca^{2+} release channels, on the concerted action of a set of proteins, which functionally and physically interact to form a complex 'molecular machine'. These proteins (i.e. triadin, junctin, homer etc.) appear to play a role in optimising Ca^{2+} release and/or in the organisation of the Ca^{2+} release molecular machinery within specific regions of the cells.

An additional level of complexity of the Ca^{2+} release molecular machinery is provided by the intracellular distribution of Ca^{2+} release channels and associated proteins, which are often organised as distinct functional domains. A better understanding of the functional significance of co-expression of Ca^{2+} release channels and the identification of other components of the Ca^{2+} release molecular machinery as well as studies on how Ca^{2+} release domains are assembled, will certainly contribute to our knowledge of the molecular basis of intracellular Ca^{2+} signalling.

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This work was supported by grants from Telethon, MURST, PAR University of Siena and ASI.

$InsP_3$ dynamics in calcium signalling

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Oscillations of cytosolic Ca^{2+} ($[Ca^{2+}]_c$) are widely observed in response to extracellular signals that generate inositol 1,4,5-trisphospate ($InsP_3$). One of the first demonstrations of this phenomenon was by Cobbold and coworkers (Woods *et al.* 1986). The mechanism underlying the generation these $[Ca^{2+}]_c$ oscillations has been the subject of much discussion (Thomas *et al.* 1996; Berridge *et al.* 1999). The predominant view is that $[Ca^{2+}]_c$ oscillations arise from positive and negative feedback effects of Ca^{2+} and $InsP_3$ on the inositol 1,4,5-trisphosphate receptor ($InsP_3R$). Thus the $InsP_3R$ acts as a Ca^{2+}-induced Ca^{2+} release channel, giving rise to regenerative cycles of Ca^{2+} release and reuptake at a fixed elevated level of intracellular $InsP_3$. While there is clear evidence that $[Ca^{2+}]_c$ oscillations can be generated in such a manner at the level of the $InsP_3R$ (Meyer & Stryer, 1988; Hirose *et al.* 1999), it appears unlikely that this can account for all of the properties of $InsP_3$-dependent $[Ca^{2+}]_c$ oscillations observed in intact, agonist-stimulated cells. For example, $[Ca^{2+}]_c$ oscillations elicited by vasopressin and α-adrenergic agonists in the liver appear as baseline-separated spikes of constant frequency, with interspike periods that can last for many minutes. One way in which the properties of $[Ca^{2+}]_c$ oscillations might be enriched, is through the interplay of multiple interacting oscillators. Of particular interest in this respect is the possibility that Ca^{2+} may have feedback effects on $InsP_3$ metabolism, to cause oscillations in the level of $InsP_3$ during agonist stimulation (Meyer & Stryer, 1988). Studies with a Plextrin Homolgy Domain-Green Fluorescent Protein construct have provided evidence for such oscillations of phospholipase activity and cytosolic $InsP_3$. It has also been suggested that other intracellular organelles or plasma membrane Ca^{2+} transport pathways may participate in the generation of $[Ca^{2+}]_c$ oscillations. In this context, mitochondria are of particular interest.

In this presentation, we will discuss some new evidence for a role of $[Ca^{2+}]_c$ feedback on $InsP_3$ metabolism in the genesis of $[Ca^{2+}]_c$ oscillations, and will revisit the role of mitochondria. In order to investigate the potential role of $InsP_3$ oscillations, we have designed a cytosolic $InsP_3$ buffer based on the ligand binding domain of the rat type I $InsP_3$ receptor (LBD), to buffer $InsP_3$ in the physiological range. This $InsP_3$ buffer is composed of green fluorescent protein fused in-frame to the N-terminal ligand-binding domain of the rat type I $InsP_3$ receptor (GFP-LBD). We demonstrate that this construct, when transiently expressed in a variety of cells, is able to suppress $[Ca^{2+}]_c$ oscillations without preventing the $InsP_3$-dependent elevation of $[Ca^{2+}]_c$. Expression of a mutated (R265Q) GFP-LBD, which is unable to bind $InsP_3$, had no effect on $[Ca^{2+}]_c$ oscillations. Taken together with direct measurements of $InsP_3$ in these cells, the data demonstrate that GFP-LBD functions as an $InsP_3$ buffer, and that $InsP_3$ oscillations are not only a consequence of $[Ca^{2+}]_c$ oscillations, but actually play a causal role in generating $[Ca^{2+}]_c$ oscillations.

Mitochondria generally maintain a relatively low level of matrix Ca^{2+} ($[Ca^{2+}]_m$), and are not considered to be a primary Ca^{2+} source for the generation of $[Ca^{2+}]_c$ signals. Nevertheless, there is much functional evidence to suggest that mitochondria are often closely associated with other Ca^{2+} mobilization pathways, including $InsP_3Rs$. Changes in $[Ca^{2+}]_m$ often parallel the spatiotemporal organization of cytosolic Ca^{2+} signals. Mitochondrial Ca^{2+} uptake has important effects on mitochondrial metabolism, which serve to co-ordinate ATP production with the activation of Ca^{2+}-dependent processes occurring in the cytoplasm. However, mitochondrial Ca^{2+} uptake can also modify the bulk flow of Ca^{2+} into the cytosol and alter the Ca^{2+} feedback effects that regulate Ca^{2+} channels in both the plasma membrane and intracellular Ca^{2+} storage compartments. Based on the properties of mitochondrial Ca^{2+} uptake and release pathways, the interactions with other Ca^{2+} mobilization pathways are believed to reflect a strategic localization of the mitochondria close to the primary Ca^{2+} channels. This apparently facilitates efficient Ca^{2+} translocation into the mitochondrial matrix, despite the relatively low $[Ca^{2+}]_c$ affinity of the mitochondrial uniporter. Mitochondrial Ca^{2+} handling

modifies the regenerative activation of $InsP_3R$ Ca^{2+} channels by Ca^{2+}, and appears to play an important role in modulating the spatial and temporal properties of $[Ca^{2+}]_c$ oscillations.

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This work was supported by funding from NIH.

All procedures accord with current National guidelines.

Regulation of the store-operated calcium current I_{CRAC}: dynamic interplay between endoplasmic reticulum, mitochondria and plasma membrane

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In electrically non-excitable cells, calcium entry is required for regulating a variety of key processes including enzyme activity, exocytosis, gene transcription and cell growth, and proliferation. The major calcium influx pathway in these cells is the store-operated one, in which the process of emptying intracellular inositol 1,4,5-trisphosphate ($InsP_3$)-sensitive calcium stores results in the activation of calcium channels in the plasma membrane. The best characterised and most widely distributed store-operated current is the calcium release-activated calcium current I_{CRAC}, which can be measured directly and unambiguously using the patch-clamp technique.

Under physiological conditions of weak intracellular calcium buffering (0.1 mM EGTA or BAPTA), receptor stimulation or simply including $InsP_3$ in the recording patch pipette fails to activate any I_{CRAC}, in spite of reducing the calcium content of the stores in rat basophilic leukaemia (RBL-1) cells. Only when the Ca^{2+} ATPase pumps on the endoplasmic reticulum (called SERCA pumps) are blocked can $InsP_3$ activate I_{CRAC} under physiological conditions. SERCA pumps are very powerful in RBL-1 cells and are able to re-sequestrate sufficient calcium back into the stores so that I_{CRAC} cannot activate. We have recently found that calcium uptake by respiring mitochondria is essential for I_{CRAC} to activate in weak buffer even when SERCA pumps are active. Removal of cytosolic calcium by mitochondria seems to compete effectively with store refilling by SERCA pumps as well as possibly reducing calcium-dependent inactivation of $InsP_3$ receptors. This enables $InsP_3$ to deplete the stores sufficiently for macroscopic I_{CRAC} to activate. Furthermore, mitochondrial calcium buffering reduces both the rate and extent of slow calcium-dependent inactivation of CRAC channels, Finally, mitochondrial calcium uptake reduces the levels of $InsP_3$ required to activate the current. By sensitising cells to lower $InsP_3$, mitochondria may determine whether relatively weak stimuli are capable of evoking store-operated calcium influx or not.

A.B.P. is a Lister Institute Senior Research Fellow.

Calcium-sensing receptors in physiology and disease

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Molecular physiology of the extracellular calcium-sensing receptor (CaR)

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The Ca^{2+}-sensing receptor (CaR) is an essential component of the calcium homeostatic system, regulating parathyroid hormone secretion, urinary calcium excretion and bone remodelling (see Brown & McLeod, 2001, for review). Initially identified from bovine parathyroid glands, CaR has been found in organs where the link with mineral ion metabolism has not been elucidated (i.e. brain, pancreas, eye, skin and many other epithelial cells). Functional studies have shown that Ca^{2+} is not the only cation capable of activating the CaR. Other di- and trivalent cations, such as Mg^{2+} and metal ions of the lanthanide series (e.g. Gd^{3+}) can all activate the CaR (Brown *et al.* 1993). In addition, polyvalent cations of various classes including endogenous compounds and pharmaco-therapeutic agents activate the CaR and may thus contribute to several pathological conditions. For instance, cationic N-terminal peptides of β-amyloid activate the CaR *in vitro* and may contribute to sustained elevations of intracellular Ca^{2+} and associated neuronal degeneration in Alzheimer's disease (Brown & MacLeod, 2001). Polycations such as the aminoglycoside antibiotic (AGA) neomycin mimic the agonist effect of high Ca_o^{2+} on the CaR (Brown *et al.* 1993). AGAs are frequently used in the treatment of gram-negative infections but are toxic to the kidney. We have recently shown that other aminoglycosides such as gentamicin and tobramycin can also activate the CaR with potencies that correlate with the net cationic charge and number of amino groups. This presentation will discuss the role of the receptor as polycation sensor, the signalling pathways evoked by different agonists and physiological effectors downstream of receptor activation.

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This work is funded by The Wellcome Trust.

Calcium signalling, the SR and smooth muscle function

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Smooth muscle occurs in many tissues and has to fulfil different roles depending upon its location, e.g. tonic force in many blood vessels and phasic activity in most viscera. While it is well understood that a rise of intracellular [Ca] is associated with smooth muscle activity, and smooth muscles have an intracellular Ca store in the SR, the precise details of Ca signalling and the role of the SR remain to be elucidated. In this presentation details of the mechanisms and the differences

between different smooth muscles will be examined in uterine and ureteric myocytes.

Most smooth muscles possess both IP_3 and ryanodine receptors on their SR membrane. It was therefore assumed that both agonist-induced, and Ca-induced Ca release (IICR and CICR, respectively) would play roles in augmenting force production. Interestingly there are few examples of CICR playing any role in this process. The coupling between Ca entry and the ryanodine receptor appears therefore to be a lot looser in smooth muscle than cardiac muscle. The role overall of the SR may differ in smooth compared with striated muscles, in that its main function appears to be as a break on contraction rather than a stimulator. For example, in uterine muscle inhibition of the SR, by emptying with cyclopiazonic acid, increases both the frequency and amplitude of contractions rather than reducing force. The Ca released from the SR appears to be vectorially targeted at surface membrane ion channels, rather than the contractile proteins. In vascular smooth muscle there is excellent evidence that SR Ca release activates K channels, and thereby contributes to vasorelaxation. This may be the mechanism operating in the uterine myocytes.

In ureteric smooth muscles we have found that there is a clear species difference in the control of the SR. Thus in rats a purely IICR mechanism operates, whereas in the guinea-pig it is a CICR system. We are exploiting this difference to explore the fundamental properties of the local release mechanisms (puffs and sparks) in the two species and how they relate to global signalling. These data will be discussed along with the corresponding electrophysiological events. We will also discuss how simultaneous direct measurements of SR and cytoplasmic [Ca] are increasing our understanding of the control of SR Ca release and its relation to cytosolic [Ca], but also raising questions concerning SR Ca availability and the relation of the SR to mitochondria.

Inositol 1,4,5-trisphosphate receptors in cardiac myocytes – where are they and what do they do?

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The role of inositol 1,4,5-trisphosphate ($InsP_3$) in cardiac myocyte function is unclear and controversial, although agonists activating $InsP_3$ generation are positive inotropic agents in the heart and have been implicated in various cardiac pathologies. We investigated the expression and subcellular localisation of $InsP_3$ receptors ($InsP_3Rs$) in rat ventricular and atrial myocytes. In addition, the consequences of activating $InsP_3Rs$ on spontaneous Ca^{2+} release were monitored using laser-scanning confocal microscopy. Rats were killed by cervical dislocation following CO_2 anaethesia according to Home Office regulations. PCR, Western blotting and $InsP_3$-binding analyses indicated that atrial and ventricular myocytes expressed $InsP_3Rs$. Both cell types mainly expressed type II $InsP_3Rs$, with atrial myocytes displaying 5-fold higher levels of $InsP_3Rs$ than ventricular cells. We observed that stimulation of atrial myocytes with $InsP_3$-generating hormones increased the likelihood of pro-arrhythmogenic events such as Ca^{2+} sparks, Ca^{2+} waves and action potentials. Direct activation of $InsP_3Rs$ by application of a membrane-permeant $InsP_3$ ester to the cells evoked similar responses, indicating that $InsP_3R$ activity alone can underlie some of the established effects of hormonal stimulation. In atrial myocytes, the predominant form of Ca^{2+} release during stimulation with hormones or $InsP_3$ esters was an increase in Ca^{2+} spark frequency. Such increases in Ca^{2+} spark activity were most commonly observed in the cellular regions where $InsP_3Rs$ and ryanodine receptors (RyRs) were co-localised. The activation of Ca^{2+} sparks by hormones and the $InsP_3$ ester suggest that cross-talk between $InsP_3Rs$ and RyRs was responsible for the enhancement of Ca^{2+} release by $InsP_3$. Our data indicate that $InsP_3Rs$ are abundantly expressed in atrial and ventricular myocytes, and that their activation can modulate cardiac function.

All procedures accord with current UK legislation.

The cardiac sarcoplasmic reticulum: relation to contraction and arrhythmias

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Cardiac contraction is initiated by an increase of cytoplasmic calcium concentration ($[Ca^{2+}]_i$); relaxation requires that $[Ca^{2+}]_i$ be reduced to control levels. Most of the calcium is released from the sarcoplasmic reticulum (SR) through a specialized released channel, the ryanodine receptor (RyR). The probability that the RyR is open is increased by a rise in $[Ca^{2+}]_i$. This therefore leads to the mechanism of calcium-induced Ca release in which the entry of a small amount of Ca into the cell through the sarcolemmal L-type Ca channels triggers the release of much more Ca through the RyR. Relaxation occurs via Ca reuptake into the SR and pumping out of the cell. There are three potential control points at which the size of the systolic Ca transient can be altered: (1) the amplitude of the L-type Ca current; (2) the properties of the RyR, in particular the relationship between $[Ca^{2+}]_i$ and channel opening; (3) the Ca content of the SR. I will discuss the importance of these various control points. A further important topic concerns the fact that when the cell and therefore the SR is excessively loaded with calcium then the SR releases Ca spontaneously. This triggers arrhythmias and ways of avoiding this will be discussed.

All procedures accord with current UK legislation.