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The versatility and complexity of calcium signalling

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Abstract. Ca^{2+} is a universal second messenger used to regulate a wide range of cellular processes such as fertilization, proliferation, contraction, secretion, learning and memory. Cells derive signal Ca^{2+} from both internal and external sources. The Ca^{2+} flowing through these channels constitute the elementary events of Ca^{2+} signalling. Ca^{2+} can act within milliseconds in highly localized regions or it can act much more slowly as a global wave that spreads the signal throughout the cell. Various pumps and exchangers are responsible for returning the elevated levels of Ca^{2+} back to the resting state. The mitochondrion also plays a critical role in that it helps the recovery process by taking Ca^{2+} up from the cytoplasm. Alterations in the ebb and flow of Ca^{2+} through the mitochondria can lead to cell death. A good example of the complexity of Ca^{2+} signalling is its role in regulating cell proliferation, such as the activation of lymphocytes. The Ca^{2+} signal needs to be present for over two hours and this prolonged period of signalling depends upon the entry of external Ca^{2+} through a process of capacitative Ca^{2+} entry. The Ca^{2+} signal stimulates gene transcription and thus initiates the cell cycle processes that culminate in cell division.

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The universality of Ca^{2+} as an intracellular messenger depends upon its enormous versatility. Many of the molecular components of the Ca^{2+} signalling system have multiple isoforms that can be mixed and matched to create a wide range of spatial and temporal signals. Ca^{2+} can operate within milliseconds in highly localized regions or it can act much more slowly as global waves of Ca^{2+} spreading throughout the cell or through large groups of cells. This versatility, which is exploited to control processes as diverse as fertilization, cell proliferation, development, secretion, chemotaxis, learning and memory must all be accomplished within the context of Ca^{2+} being a highly toxic ion. If its normal spatial and temporal boundaries are exceeded, this deregulation of Ca^{2+} signalling results in cell death through both necrosis and apoptosis. The aim of this review is twofold: I will first describe the complex nature of Ca^{2+} signalling

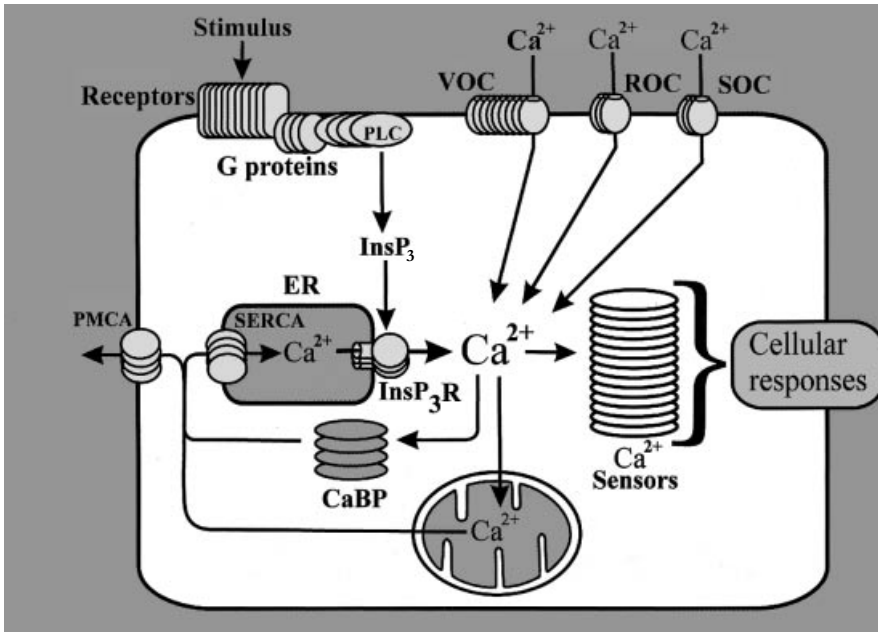


FIG. 1. The Ca^{2+} signalling toolkit. All of the molecular components regulating the Ca^{2+} signalling pathway are composed of multiple components, often closely related isoforms with subtly different properties. The duplication shown on the figure illustrates the degree of this diversity. Inositol 1,4,5-trisphosphate ($InsP_3$), which is generated by phospholipase C (PLC), acts on $InsP_3$ receptors ($InsP_3R$) located on the endoplasmic reticulum (ER). Ca^{2+} enters the cell through multiple isoforms of voltage-operated channels (VOCs), receptor-operated channels (ROCs) and store-operated channels (SOCs). Various Ca^{2+}-binding proteins (CaBPs) buffer Ca^{2+} both in the cytoplasm and within the lumen of the ER. Ca^{2+} is pumped out of the cell by exchangers and plasma membrane Ca^{2+}-ATPases (PMCA) or into the ER by sarcoendoplasmic reticulum Ca^{2+}-ATPases (SERCA).

and then consider how this messenger system functions in cell proliferation and cell death.

The Ca^{2+} signalling network

The hallmark of Ca^{2+} signalling is its complexity. One manifestation of this is the existence of two separate sources of Ca^{2+}, which can be derived from either internal stores or by uptake from the external medium (Fig. 1). Different channels and pumps regulate each source. Signalling begins when the external stimulus binds to receptors that either activate channels in the plasma membrane or generate Ca^{2+}-mobilizing signals that release Ca^{2+} from the internal stores. The Ca^{2+} that flows into the cytoplasm functions as a messenger to stimulate numerous

Ca^{2+}-sensitive processes. Finally, there are OFF mechanisms, composed of pumps and exchangers, which remove Ca^{2+} from the cytoplasm to restore the resting state. Most of the processes of the signalling pathway are carried out by different components, which means that each cell has access to a diverse molecular toolkit (Fig. 1). By mixing and matching all the available possibilities, cells can create Ca^{2+} signals with widely different spatial and temporal properties.

Generation of Ca^{2+} signals

There are families of Ca^{2+} entry channels defined by the way in which they are activated. We know most about voltage-operated channels (VOCs) of which there are at least 10 types (Fig. 1) with subtly different properties. Ca^{2+} can also enter cells through receptor-operated channels (ROCs) and through store-operated channels (SOCs). There is considerable debate as to how empty stores can activate channels in the plasma membrane. Recent evidence has begun to support a conformational-coupling mechanism, which proposed that the inositol-1,4,5-trisphosphate ($InsP_3$) receptors in the plasma membrane are directly coupled to the SOCs in the plasma membrane (Berridge et al 2000). There is considerable interest in these SOCs since they provide the Ca^{2+} signal that controls cell proliferation (see later).

Signal Ca^{2+} is also derived from the internal stores using channels regulated by Ca^{2+}-mobilizing messengers, such as $InsP_3$ that diffuses into the cell to engage the $InsP_3$ receptors ($InsP_3Rs$) that release Ca^{2+} from the endoplasmic reticulum (ER). Cyclic ADP ribose (cADPR) acts by releasing Ca^{2+} from ryanodine receptors (RYRs). Sphingosine-1-phosphate (S1P) and nicotinic acid dinucleotide phosphate (NAADP) release Ca^{2+} by binding to channels that have yet to be characterized.

Most attention has focused on the $InsP_3Rs$ and the RYRs, which are regulated by a number of factors — the most important of which is Ca^{2+} itself. For example, the $InsP_3Rs$ have a bell-shaped Ca^{2+} dependence in that low concentrations (100–300 nM) are stimulatory but above 300 nM, Ca^{2+} becomes inhibitory and acts to switch the channel off. Once the receptor binds $InsP_3$, it becomes sensitive to the stimulatory action of Ca^{2+}. In the same way, cADPR increases the Ca^{2+} sensitivity of the RYRs. The $InsP_3Rs$ and the RYRs have a mechanism of *Ca^{2+}-induced Ca^{2+} release* (CICR) and this autocatalytic process enables individual channels to communicate with each other to establish highly coordinated Ca^{2+} signals often organized into propagating waves. The main function of the Ca^{2+}-mobilizing messengers, therefore, is to alter the sensitivity of the $InsP_3Rs$ and RYRs to this stimulatory action of Ca^{2+}.

These different Ca^{2+}-mobilizing messengers often coexist in cells where they seem to be controlled by different receptors. For example, in the exocrine

pancreas, acetylcholine receptors act through $InsP_3$ whereas CCK receptors employ NAADP and cADPR (Cancela et al 1999). Similarly, human SH-SY5Y cells have acetylcholine receptors linked through $InsP_3$ while lysophosphatidic acid (LPA) acts through S1P (Young et al 1999). The complexity of the signalling network is thus enhanced by having different Ca^{2+}-mobilizing messengers linked to separate input signals.

Ca^{2+}-sensitive processes

Various Ca^{2+}-sensitive processes translate Ca^{2+} signals into cellular responses. There are a large number of Ca^{2+}- binding proteins, which can be divided into Ca^{2+} sensors and Ca^{2+} buffers. The Ca^{2+} sensors respond to the increase in Ca^{2+} by activating a wide range of responses. Classical examples of sensors are troponin C (TnC) and calmodulin, which have four E-F hands that bind Ca^{2+} and undergo a pronounced conformational change to activate a variety of downstream effectors. TnC has a somewhat limited function of controlling the interaction of actin and myosin during the contraction cycle of cardiac and skeletal muscle. By contrast, calmodulin is used much more generally to regulate many different processes such as the contraction of smooth muscle, cross-talk between signalling pathways, gene transcription, ion channel modulation and metabolism. The same cell can use different detectors to regulate separate processes. In skeletal muscle, for example, TnC regulates contraction whereas calmodulin stimulates phosphorylase thereby ensuring an increase in ATP production.

In addition to the above proteins, which have a more general function, there are a large number of Ca^{2+}-binding proteins designed for more specific functions. For example, synaptotagmin is associated with membrane vesicles and is responsible for mediating exocytosis. A large family of S100 Ca^{2+}-binding proteins seems to be particularly important in cell proliferation and have been implicated in cancer growth and metastasis. For example, human chromosome Iq21 has a cluster of approximately 10 S100 genes that are differentially expressed in neoplastic tissues. Melanoma cells overexpress S100B and antibodies against this Ca^{2+}-binding protein are used for tumour typing and diagnosis of melanoma. S100B can activate a nuclear serine/threonine protein kinase (Millward et al 1998) and can cooperate with protein kinase C to induce the translocation of p53 early in the G1 phase of the cell cycle (Scotto et al 1999).

Once Ca^{2+} has carried out its signalling functions, it is rapidly removed from the cytoplasm by various pumps and exchangers located both on the plasma membrane and on the internal stores (Fig. 1). The mitochondrion is another important component of the OFF mechanism in that it sequesters Ca^{2+} rapidly during the recovery phase and then slowly releases it back when the cell is at rest. In order to

synthesize ATP, the mitochondrion extrudes protons to create the electrochemical gradient that is used to synthesize ATP. Exactly the same gradient is used to drive Ca^{2+} uptake through a uniporter which functions much like a channel. The mitochondrion has a large capacity to accumulate Ca^{2+}. Once the cytosolic level of Ca^{2+} has returned to its resting level, a Na^+/Ca^{2+} exchanger transfers the large load of Ca^{2+} back into the cytoplasm where it is once again returned to the ER or removed from the cell. In addition to this slow efflux pathway, Ca^{2+} can also leave through a permeability transition pore (PTP). This PTP may have two functional states. First, there is a low conductance state that acts reversibly, allowing mitochondria to become excitable and thus contributing to the generation of Ca^{2+} waves (Ichas et al 1997). Second, there is an irreversible high conductance state of the PTP that has a dramatic effect on the mitochondrion in that it collapses the transmembrane potential and leads to the release of cyctochrome c and the initiation of apoptosis.

During normal signalling, therefore, there is a continuous ebb and flow of Ca^{2+} between the ER and the mitochondria. At the onset of each spike, a small bolus of Ca^{2+} is released to the cytoplasm and some of this signal enters the mitochondria where it has a temporary residence before being returned to the ER. Mitochondria contribute to the onset of apoptosis if this normal exchange of Ca^{2+} with the ER is distorted.

The apoptosis regulatory proteins that function either as death antagonists (Bcl2 and $BclX_L$) or death agonists (Bax, Bak and Bad), may exert some of their actions by interfering with the Ca^{2+} dynamics of these two organelles. For example, Bax and Bad accelerate the opening of the voltage-dependent anion channel, which is part of the PTP, and thus contribute to the release of cytochrome c (Shimizu et al 1999). On the other hand, Bcl2 and $BclX_L$ seem to act by blocking Ca^{2+}-induced apoptosis, enabling the mitochondria to cope with large loads of Ca^{2+} (Zhu et al 1999). Bcl2 is also present on the ER where it acts to enhance the store of Ca^{2+} (Zhu et al 1999) perhaps by up-regulating the expression of Ca^{2+} pumps (Kuo et al 1998).

Spatial and temporal aspects of Ca^{2+} signalling

Much of the versatility of Ca^{2+} signalling arises from the way that it is presented in both time and space. Our understanding of the spatial aspects has increased enormously due to advances in imaging technology that have enabled us to visualize the elementary events of Ca^{2+} signalling. These elementary events are the basic building blocks of Ca^{2+} signals in that they represent the Ca^{2+} that results from the opening of either single or small groups of channels. They have been described most extensively for the channels that release Ca^{2+} from the internal stores. Whether or not these channels open to release Ca^{2+} is determined by their degree of excitability, which is controlled by a number of factors. As described

earlier, the primary determinant for the $InsP_3Rs$ is $InsP_3$ whereas the RYRs are sensitive to cADPR. Both channels are also sensitive to the degree of Ca^{2+} loading in the store.

At low levels of stimulation, the level of excitability is such that individual RYRs or $InsP_3Rs$ open; such events have been recorded as quarks or blips, respectively. They may be considered as the fundamental events that form the basis of most Ca^{2+} signals. These single-channel events are rare and the more usual event is somewhat larger, resulting from the coordinated opening of small groups of $InsP_3Rs$ or RYRs known as puffs or sparks, respectively. Sparks were first described in cardiac cells where they arise from a group of RYR2 channels opening in response to Ca^{2+} entering through L channels. Puffs have a wide range of amplitudes suggesting that there are variable numbers of $InsP_3Rs$ within each cluster. These sparks and puffs are the elementary events of Ca^{2+} signalling that contribute to the intracellular waves that sweep through cells to create global Ca^{2+} signals. When gap junctions connect cells, such intracellular waves can spread to neighbouring cells thus creating intercellular waves capable of coordinating the activity of large groups of cells.

In addition to creating global responses, these elementary events have another important function in that they can carry out signalling processes within highly localized cellular domains. A classic example is the process of exocytosis at synaptic endings where N- or P/Q-type VOCs create a local pulse of Ca^{2+} to activate synaptotagmin to trigger vesicle release. Sparks located near the plasma membrane of excitable cells activate Ca^{2+}-sensitive K^+ channels bringing about membrane hyperpolarization, which can regulate the excitability of neurons or the contractility of smooth muscle cells. In HeLa cells, Ca^{2+} puffs are concentrated around the nucleus where they feed Ca^{2+} directly into the nucleoplasm (Lipp et al 1997). Finally, as mentioned earlier, the mitochondria located near the sites of elementary events take up Ca^{2+} rapidly and this stimulates mitochondrial metabolism to increase the formation of ATP.

In addition to these spatial variations, there are also marked differences in the temporal aspect of Ca^{2+} signalling. More often than not, Ca^{2+} signals are presented as brief spikes. In some cases, individual spikes are sufficient to trigger a cellular response as occurs during contraction of skeletal or cardiac muscle or the release of synaptic vesicles by exocytosis. When longer periods of signalling are necessary, such spikes are repeated to give oscillations with widely differing frequencies. Periods within the 1–60 second range are found in the pancreas and liver, but much longer periods of 1–5 minutes have been recorded in mammalian eggs following fertilization. A Ca^{2+} oscillator that initiates mitosis during the cell cycle has an even longer period of signalling of approximately 24 hours.

The mitotic Ca^{2+} oscillator is particularly interesting because it is an integral component of the control mechanisms that regulate the cell cycle. The latter is an

orderly programme of events controlled by two linked oscillators: a cell cycle oscillator and the Ca^{2+} oscillator (Swanson et al 1997). The former depends upon the synthesis and periodic proteolysis of various cyclins at specific points during the cell cycle. The Ca^{2+} oscillator, based on the periodic release of stored Ca^{2+}, is responsible for initiating specific events associated with mitosis such as nuclear envelope breakdown (NEBD), anaphase and cell cleavage. As the one-cell mouse embryo approaches its first mitosis, there are a series of spontaneous Ca^{2+} transients responsible for triggering various events during mitosis such as NEBD, anaphase and cleavage to the two-cell stage (Chang & Meng 1995). Just what drives the Ca^{2+} oscillator is a mystery but there are indicators that it depends upon the periodic elevation of $InsP_3$. In the case of the sea urchin, the level of $InsP_3$ is increased at distinct points during mitosis such as NEBD, anaphase and cleavage, at the time of each spontaneous Ca^{2+} transient (Ciapa et al 1994).

When cells need to be activated for prolonged periods, a single Ca^{2+} spike is not sufficient and is replaced by Ca^{2+} oscillations. Cells respond to changes in stimulus intensity by varying spike frequency. Such frequency-modulated signalling is used to control processes such as liver metabolism, smooth muscle contractility and differential gene transcription, especially in developing systems. For example, presenting Ca^{2+} in the form of spikes was more effective in initiating gene expression than a steady maintained level of Ca^{2+} (Li et al 1998). A low frequency of spiking activated NF-κB, whereas higher frequencies were necessary to switch on NF-AT and Oct (Dolmetsch et al 1998). Such oscillatory activity is particularly important for the development of both neural and muscle cells (Buonanno & Fields 1999). In *Xenopus*, spontaneous Ca^{2+} spikes produced by RYRs during a narrow developmental window are responsible for the differentiation of myocytes into somites (Ferrari et al 1998). Neural development is also mediated by Ca^{2+} spikes that control process such as differentiation (Gu & Spitzer 1997, Carey & Matsumoto 1999), the behaviour of growth cones (Gomez & Spitzer 1999) and the establishment of the specific connections within neural circuits (Feller 1999).

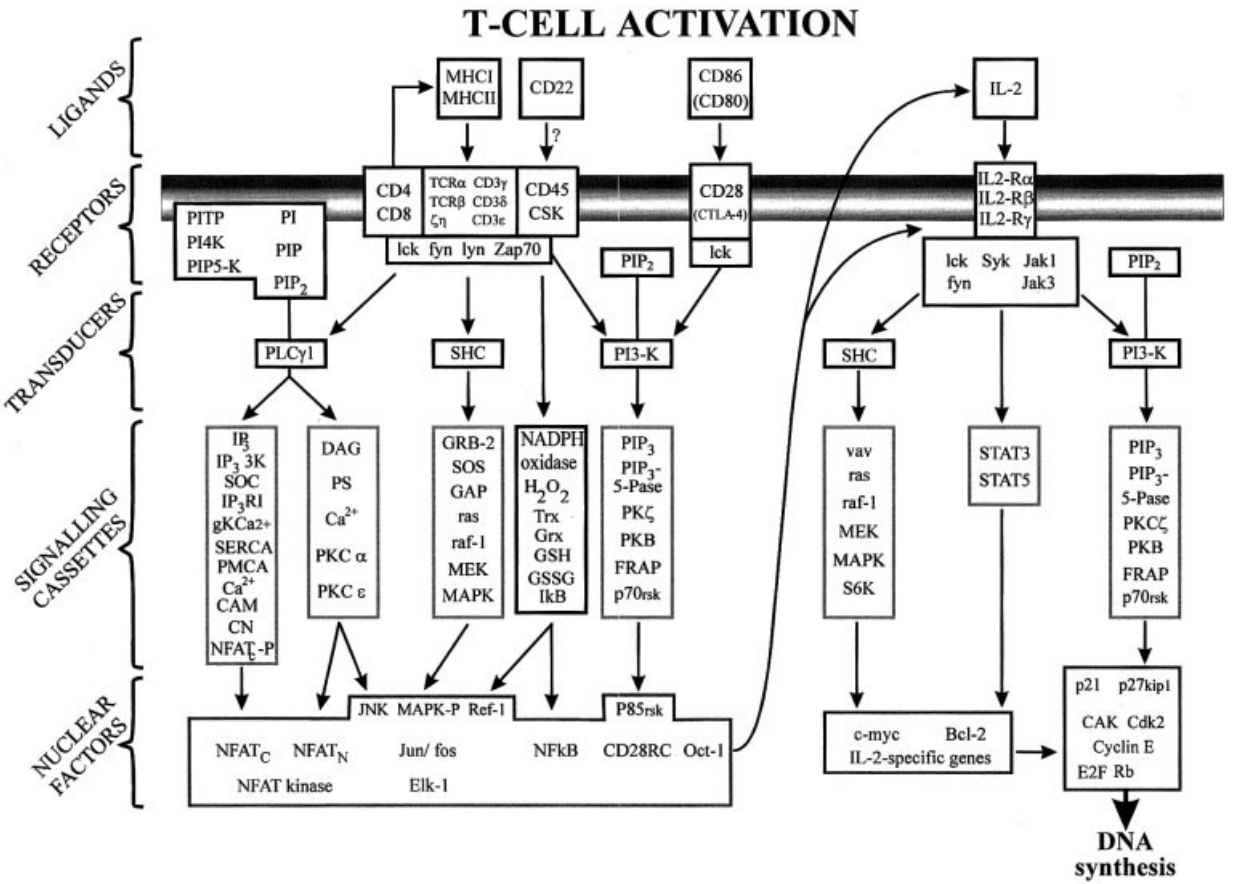
In order to use such a frequency-modulated signalling system, cells have evolved sophisticated 'molecular machines' for decoding such frequency encoded Ca^{2+} signals. The two Ca^{2+}-sensitive proteins that seem to play a role in decoding are CaM kinase II (DeKoninck & Schulman 1998) and protein kinase C (Oancea & Meyer 1999).

Cell proliferation

A good example of the complexity of Ca^{2+} signalling is its role in regulating cell proliferation. Once cells have differentiated to perform specific functions, they usually stop proliferating. In many cases, however, such differentiated cells maintain the option of returning to the cell cycle and this usually occurs in

response to growth factors. For example, lymphocytes proliferate rapidly in response to antigens, smooth muscle cells respond to growth factors such as PDGF at the sites of wounds and astrocytes are stimulated to grow at sites of brain injury. In many of these examples, Ca^{2+} is one of the key regulators of cell proliferation, where it functions in conjunction with other signalling pathways such as those regulated through MAP kinase and phosphatidylinositol-3 kinase (PI 3-K) (Lu & Means 1993, Berridge 1995). The function of Ca^{2+} in regulating cell proliferation is well illustrated in lymphocytes responding to antigen. Figure 2 attempts to summarize all the signalling elements that are used by a T cell as it responds to the arrival of an antigen. In this case, the antigen functions as a 'growth factor' that binds to the T cell receptor to initiate the assembly of a 'supramolecular activation cluster' (Monks et al 1998) containing scaffolding and signal transducing elements. The latter function to relay information into the nucleus using various signalling cassettes. The cassettes linked to phospholipase C (PLC)γ1, which produces both diacylglycerol (DAG) and $InsP_3$, are particularly important and frequently associated with the action of growth factors and have been implicated in cell transformation. In fact, PLCγ has been referred to as a malignancy linked signal transducing enzyme (Yang et al 1998) and its overexpression will promote transformation and tumorigenesis in NIH 3T3 cells (Smith et al 1998). The $InsP_3$ formed by PLCγ1 releases Ca^{2+} from the internal store, which then promotes entry of external Ca^{2+} through a SOC.

When used for controlling cell proliferation, this Ca^{2+} signalling pathway needs to be active for a prolonged period — two hours in the case of lymphocytes. Since the stores have a very limited capacity, this prolonged period of Ca^{2+} signalling is critically dependent on this influx of external Ca^{2+}. There are two modulatory mechanisms that function to maintain Ca^{2+} signalling (Fig. 3). The first is an example of the cross talk between signalling pathways and concerns the ability of PI 3-K to stimulate PLCγ1 to maintain the supply of $InsP_3$ (Scharenburg & Kinet 1998). Formation of the lipid second messenger PIP_3 activates the non-receptor tyrosine kinase Btk that then phosphorylates and activates PLCγ1. The tumour suppressor PTEN, which acts as a 3-phosphatase to lower the level of phosphatidylinositol-3,4,5-trisphosphate (PIP_3), reduces both the level of $InsP_3$ and the influx of external Ca^{2+} (Morimoto et al 2000). The second is the activation of potassium channels that serve to hyperpolarize the membrane which is essential to maintain the entry of external Ca^{2+} (Lewis & Cahalan 1995). For example, cell proliferation is regulated by IK_{Ca}, which is inhibited by charybdotoxin and iberiotoxin. The net effect of these two mechanisms is to ensure a continuous influx of external Ca^{2+}, which seems to be one of the principle early signals to promote cell proliferation. A Ca^{2+} influx inhibitor carboxy-amidotriazole can prevent cell proliferation and has been used in clinical trials to control refractory cancers (Kohn et al 1996).



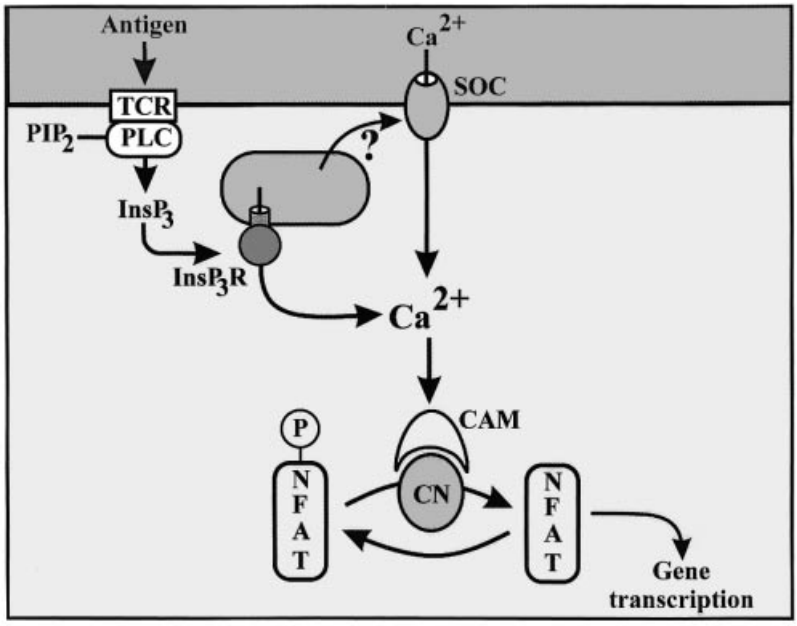


FIG. 3. The role of Ca^{2+} in lymphocyte activation. The antigen activates the T cell receptor (TCR), which stimulates phospholipase C (PLC) to hydrolyse phosphatidylinositol-4,5-bisphosphate (PIP_2), to release inositol-1,4,5-trisphosphate ($InsP_3$) to the cytosol. $InsP_3$ releases Ca^{2+} from the internal store, which then sends an unknown signal to the store-operated channels (SOCs) in the plasma membrane. Ca^{2+} acts through calmodulin (CAM) to stimulate calcineurin (CN) which dephosphorylates the nuclear factor of activated T cells (NFAT) enabling it to enter the nucleus to initiate gene transcription.

The main function of Ca^{2+} is to activate transcription factors either in the cytoplasm (NF-AT, NF-κB) or within the nucleus (CREB). The role of Ca^{2+} in stimulating gene transcription is very similar in neurons undergoing learning as it is in cells being induced to grow. An increase in Ca^{2+} is one of the signals capable of bringing about the hydrolysis of the inhibitory IκB subunit allowing the active NF-κB subunit to enter the nucleus. Perhaps the most important action of Ca^{2+} is to stimulate calcineurin to dephosphorylate NF-AT, which then enters the nucleus (Fig. 3) (Crabtree 1999). As soon as Ca^{2+} signalling ceases,

FIG. 2. A spatiotemporal map of T cell activation. The spatial aspect (i.e. from top to bottom) concerns the way in which the antigen binding to the complex T cell receptor activates a number of signalling cassettes that transfer information from the plasma membrane to the nucleus. The temporal aspect (i.e. from left to right) deals with the flow of information through the sequential signalling elements that occurs during the protracted G1 period of the cell cycle and culminates in the activation of either proliferation or apoptosis.

kinases in the nucleus rapidly phosphorylate NF-AT which then leaves the nucleus and transcription ceases. The prolonged period of Ca^{2+} signalling that is required for proliferation to occur is thus necessary to maintain the transcriptional activity of NF-AT. Transcription is inhibited in mutants with defective SOCs that cannot sustain Ca^{2+} signalling (Timmerman et al 1996). Likewise, the immunosuppressant drugs cyclosporin A and FK506 prevent transcription by inhibiting the action of calcineurin. In contrast to the previous two transcription factors that are activated within the cytoplasm, CREB is a nuclear Ca^{2+}-responsive element, which is phosphorylated by CaMKII and CaMKIV. In addition, Ca^{2+} acting within the nucleus is also responsible for stimulating the Ca^{2+}-sensitive transcriptional coactivator CREB-binding protein (CBP) (Hardingham et al 1999). A CaM inhibitory peptide targeted to the nucleus was able to block DNA synthesis and cell cycle progression thus emphasizing the importance of a nuclear Ca^{2+} signal for cell proliferation (Wang et al 1996). These transcription factors activate a large number of target genes, some code for progression factors such as the interleukin 2 system responsible for switching on DNA synthesis whereas others produce components such as Fas and the Fas ligand that are responsible for apoptosis (Fig. 2). Ca^{2+} thus plays a central role in putting in place the signalling systems that enable cells to decide whether to grow or to die.

References

Berridge MJ 1995 Calcium signalling and cell-proliferation. Bioessays 17:491–500

Berridge MJ, Lipp P, Bootman MD 2000 Signal transduction. The calcium entry pas de deux. Science 287:1604–1605

Buonanno A, Fields RD 1999 Gene regulation by patterned electrical activity during neural and skeletal muscle development. Curr Opin Neurobiol 9:110–120

Cancela JM, Churchill GC, Galione A 1999 Coordination of agonist-induced Ca^{2+}-signalling patterns by NAADP in pancreatic acinar cells. Nature 398:74–76

Carey B, Matsumoto SG 1999 Spontaneous calcium transients are required for neuronal differentiation of murine neural crest. Dev Biol 215:298–313

Chang DC, Meng CL 1995 A localized elevation of cytosolic-free calcium is associated with cytokinesis in the zebrafish embryo. J Cell Biol 131:1539–1545

Ciapa B, Pesando D, Wilding M, Whitaker M 1994 Cell-cycle calcium transients driven by cyclic changes in inositol trisphosphate levels. Nature 368:875–878

Crabtree GR 1999 Generic signals and specific outcomes: signaling through Ca^{2+}, calcineurin, and NF-AT. Cell 96:611–614

De Koninck P, Schulman H 1998 Sensitivity of CaM kinase II to the frequency of Ca^{2+} oscillations. Science 279:227–230

Dolmetsch RE, Xu KL, Lewis RS 1998 Calcium oscillations increase the efficiency and specificity of gene expression. Nature 392:933–936

Feller MB 1999 Spontaneous correlated activity in developing neural circuits. Neuron 22: 653–656

Ferrari MB, Ribbeck K, Hagler DJ, Spitzer NC 1998 A calcium signaling cascade essential for myosin thick filament assembly in *Xenopus* myocytes. J Cell Biol 141:1349–1356

Gomez TM, Spitzer NC 1999 *In vivo* regulation of axon extension and pathfinding by growth-cone calcium transients. Nature 397:350–355

Gu XN, Spitzer NC 1997 Breaking the code: regulation of neuronal differentiation by spontaneous calcium transients. Dev Neurosci 19:33–41

Hardingham GE, Chawla S, Cruzalegui FH, Bading H 1999 Control of recruitment and transcription-activating function of CBP determines gene regulation by NMDA receptors and L-type calcium channels. Neuron 22:789–798

Ichas F, Jouaville LS, Mazat JP 1997 Mitochondria are excitable organelles capable of generating and conveying electrical and calcium signals. Cell 89:1145–1153

Kohn EC, Reed E, Sarosy G et al 1996 Clinical investigation of a cytostatic calcium influx inhibitor in patients with refractory cancers. Cancer Res 56:569–573

Kuo TH, Kim HRC, Zhu LP, Yu YJ, Lin HM, Tsang W 1998 Modulation of endoplasmic reticulum calcium pump by Bcl-2. Oncogene 17:1903–1910

Lewis RS, Cahalan MD 1995 Potassium and calcium channels in lymphocytes. Annu Rev Immunol 13:623–653

Li WH, Llopis J, Whitney M, Zlokarnik G, Tsien RY 1998 Cell-permeant caged $InsP_3$ ester shows that Ca^{2+} spike frequency can optimize gene expression. Nature 392:936–941

Lipp P, Thomas D, Berridge MJ, Bootman MD 1997 Nuclear calcium signalling by individual cytoplasmic calcium puffs. EMBO J 16:7166–7173

Lu KP, Means AR 1993 Regulation of the cell cycle by calcium and calmodulin. Endocr Rev 14:40–58

Millward TA, Heizmann CW, Schafer BW, Hemmings BA 1998 Calcium regulation of Ndr protein kinase mediated by S100 calcium-binding proteins. EMBO J 17:5913–5922

Monks CRF, Freiberg BA, Kupfer H, Sciaky N, Kupfer A 1998 Three-dimensional segregation of supramolecular activation clusters in T cells. Nature 395:82–86

Morimoto AM, Tomlinson MG, Nakatani K, Bolen JB, Roth RA, Herbst R 2000 The MMAC1 tumor suppressor phosphatase inhibits phospholipase C and integrin-linked kinase-activity. Oncogene 19:200–209

Oancea E, Meyer T 1998 Protein kinase C as a molecular machine for decoding calcium and diacylglycerol signals. Cell 95:307–318

Scharenberg AM, Kinet JP 1998 PtdIns-3,4,5-P_3: a regulatory nexus between tyrosine kinases and sustained calcium signals. Cell 94:5–8

Scotto C, Delphin C, Deloulme JC, Baudier J 1999 Concerted regulation of wild-type p53 nuclear accumulation and activation by S100B and calcium-dependent protein kinase C. Mol Cell Biol 19:7168–7180

Shimizu S, Narita M, Tsujimoto Y 1999 Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. Nature 399:483–487

Smith MR, Court DW, Kim HK et al 1998 Overexpression of phosphoinositide-specific phospholipase Cγ in NIH 3T3 cells promotes transformation and tumorigenicity. Carcinogenesis 19:177–185

Swanson CA, Arkin AP, Ross J 1997 An endogenous calcium oscillator may control early embryonic division. Proc Natl Acad Sci USA 94:1194–1199

Timmerman LA, Clipstone NA, Ho SN, Northrop JP, Crabtree GR 1996 Rapid shuttling of NF-AT in discrimination of Ca^{2+} signals and immunosuppression. Nature 383:837–840

Wang JH, Moreira KM, Campos B, Kaetzel MA, Dedman JR 1996 Targeted neutralization of calmodulin in the nucleus blocks DNA synthesis and cell cycle progression. Biochim Biophys Acta 1313:223–228

Yang H, Shen F, Herenyiova M, Weber G 1998 Phospholipase C (EC 3.1.4.11): a malignancy linked signal transduction enzyme. Anticancer Res 18:1399–1404

Young KW, Challiss RAJ, Nahorski SR, Mackrill JJ 1999 Lysophosphatidic acid-mediated Ca^{2+} mobilization in human SH-SY5Y neuroblastoma cells is independent of phosphoinositide signalling, but dependent on sphingosine kinase activation. Biochem J 343:45–52

Zhu LP, Ling S, Yu XD et al 1999 Modulation of mitochondrial Ca^{2+} homeostasis by Bcl-2. J Biol Chem 274:33267–33273

DISCUSSION

Fields: You said something that appears contradictory. You said that subthresold events such as low agonist concentrations lead to local Ca^{2+} changes, and then higher intensity stimulation leads to a global Ca^{2+} response. Then you also told us that the local Ca^{2+} changes in spines are important for long-term potentiation (LTP). I would be interested in your comments on this. The normal stimulus that induces LTP causes the neuron to fire action potentials and therefore causes a global change in Ca^{2+}. How can the importance of local Ca^{2+} change be reconciled with situations where the stimulus would produce a global Ca^{2+} change?

Berridge: In the case of LTP one has to be very careful in terms of understanding local versus global effects. In the case of the spine, Ca^{2+} is input specific in the sense that it is elevated in only those synapses that are active. In addition, you can get global changes, but my feeling is that the concentration caused by a global Ca^{2+} change within the whole dendritic tree will probably not reach threshold to modify individual spines. Neurons have an enormous concentration of Ca^{2+} buffers and they vary in the proportion of these buffers that are expressed. One of the functions of such buffers is to dampen out Ca^{2+} signals. Every time the neuron fires, as part of its information-processing role, there is a back-propagating action potential that spreads into the dendritic tree, resulting in a global Ca^{2+} change. You don't want to modify your synapses every time you are processing information. Although this is a global Ca^{2+} change, I would argue that the buffers ensure that the concentration is relatively low within individual spines. However, if you have a back-propogated action potential occurring in conjunction with the activation of a synapse, then there will be a much larger but localized elevation of Ca^{2+} within the spine.

Fields: Are there measurements that indicate that the Ca^{2+} rise is insulated or augmented in the spine?

Berridge: The measurements are only really just starting, but we already have recordings to show that Ca^{2+} signals can be restricted to individual spines. It is amazing that we are actually able to measure the Ca^{2+} in these spines: the estimate is that at rest there are only six free Ca^{2+} ions in each spine.

Sejnowski: We have done simulation of Ca^{2+} entry into the spines of pyramidal cells. You have to be careful interpreting these pictures, because the Ca^{2+} indicator is itself a buffer.

Noble: I love the emphasis on beauty rather complexity. It is the unravelling of these beautiful systems that is the great joy to those of us who are trying to work at higher levels. Adding to your versatility, there is at least one cell for which the *amplitude* of the global signal is variable in an important way, which is the heart. One of the reasons I say this is connected with my comment on the joy of unravelling complex systems: it was actually quite difficult to get a graded release in modelling the Ca^{2+} release mechanism in the heart. I would even go so far to say that although we have now got models of this, we still don't fully understand why it is as graded as it is.

Berridge: Recent studies on Ca^{2+} sparks in cardiac cells have provided an explanation for such graded responses. This has been one of the unsolved problems in physiology: how can a process of Ca^{2+}-induced Ca^{2+} release (CICR), which is a positive feedback mechanism, generate a graded release of Ca^{2+}? One of the ideas is that the gradation may actually depend upon the variable recruitment of these elementary events.

Noble: Exactly. You may need to bring in what you call the 'physiological toolkit'. Trying to model with just one variable of free Ca^{2+} is only possible provided you make Ca^{2+} do two things: activate the release and also inactivate it with another time course. People who have tried to isolate that inactivation process by breaking the system down into its component bits can't find it. There has to be something else, and I have a strong suspicion it may be in what you call the physiological toolkit: the way Ca^{2+} is located in complex physiological spaces and structures.

Berridge: To expand on this idea of the physiological toolkit, it looks very much as if at low depolarizations, relatively few of these individual events are recruited. Each of these spark sites is an autonomous unit that fires independently of the others. Each unit is using CICR, but because they are separated from each other Ca^{2+} doesn't spread to neighbouring sites. By varying the level of depolarization, you can recruit variable numbers of these individual events. By having autonomous units it is possible to get a graded response. It is a very elegant solution.

Schultz: You mentioned the fact that cADP ribose stimulates the Ca^{2+} release and that cGMP stimulates cADP ribose formation. In which systems is this control important? I know systems in which cGMP blocks Ca^{2+} release and others where it stimulates Ca^{2+} influx, but I don't really know of a system in which cGMP would stimulate Ca^{2+} release.

Berridge: That's a good point. This was a very old slide which came from work on the sea urchin, where there seems to be some indication of cGMP playing a role in fertilization. The idea is that fertilization may generate cADP ribose, which in the sea urchin is responsible for activating Ca^{2+} release from the ER. In other cell types, there is some uncertainty concerning cADP ribose with regard to its precise function. There's evidence that it is playing an important role in the pancreas,

which is activated by different hormonal systems. Acetylcholine acts through the $InsP_3$ system, whereas CCK seems to use cADP ribose.

Sejnowski: I liked your idea about looking at spatial scales. You used the micron scale as a convenient one. I'd like to suggest that there is actually a sub-micron scale that is equally important, especially in synapses, because of the fact that much of the machinery of the receptors and the Ca^{2+}–calmodulin complexes are right there under the plasma membrane, organized in a very precise way. In fact, these are little machines: they are really complexes. They are positioned such that when Ca^{2+} does enter, say through the NMDA receptor, it is at a very high concentration, briefly and locally. It could be that there is an even more precise molecular machinery.

Berridge I agree that we need to study more closely the precise morphology and molecular organization of the spine. There's some intriguing evidence coming out in terms of the variation between, for example, the CA1 neuron and the Purkinje cell in terms of the distribution of the metabotropic receptors, the G_q/G_{11} transducers and various phospholipase C isoforms.

Segel: You mentioned that contraction and relaxation occur together in smooth muscle. I have thought for a long time that there must be some sort of system that makes smooth muscle contract smoothly, in the sense of coordinating all the sub-parts of it. Has that been studied? Is there such a system where you would need both contraction and relaxation together to make everything work in a coordinated fashion?

Berridge: There is not just one type of smooth muscle, but instead there is an enormous variety of types. A lot of these smooth muscle cells function in a tonic state of contraction: they are poised between contraction and relaxation. This dynamic equilibrium between relaxation and contraction may be governed by the spatial organization of the Ca^{2+} signalling system. Günter Schultz is an expert on smooth muscle: perhaps he would like to comment on this?

Schultz: With regard to the tonic aspect of smooth muscle contraction, the Ca^{2+}-independent pathway causes contraction via G_{12} and G_{13} signalling to Rho and Rho kinase, and inhibition of myosin phosphatase. This is an important aspect, contributing as much as the Ca^{2+} part does to the overall contraction.

Laughlin: If one sat down with a piece of paper and decided to design a signalling system in a cell which depended on the propagation of some signalling molecule, one probably would not choose a molecule that was very heavily buffered. Because Ca^{2+} is heavily buffered, the density of sites required to regenerate and propagate the Ca^{2+} signal must be quite high. Have you any idea what that density is? How many of these elementary sites do you need in order to propagate a wave through the cell?

Berridge: That's an interesting question. In the case of muscle it might be possible to find that out, and there is some evidence for a high density of RYRs. But in other

cell types we don't really have that information. It's quite hard to do immunohistochemistry on, say, $InsP_3$ receptors, although this is something we would like to know because the density of these sites has a marked effect in determining the rate of propagation. In fact, nature has done some beautiful experiments for us. For example, in the *Xenopus* oocyte the fertilization wave progresses faster at the animal pole than at the vegetal pole. This correlates with the density of $InsP_3$ receptors, which is much higher at the animal pole where the wave moves quickly.

Schöfl: I have a few questions regarding the localized Ca^{2+} puffs around the nucleus. Do they also occur at rest, which might be important for the control of Ca^{2+}-dependent genes expressed in the basal state? Are these Ca^{2+} puffs differentially regulated by distinct agonists? Is there any evidence that for example $InsP_3$-mobilizing agonists are better at enhancing these perinuclear changes in Ca^{2+} than agonists which predominantly activate Ca^{2+} influx through voltage-gated Ca^{2+} channels?

Berridge: With regard to the first point, we do see some activity at rest, but it is very low. We need to activate the cell to see these elementary events. It might just be fortuitous that we find these all around the nucleus because that's where most of the endoplasmic reticulum is located. The ER also spreads out into the periphery, but we don't see many puffs out there. It's a paradoxical situation in that the peripheral ER is located closer to the site of $InsP_3$ generation, yet when you start to activate the cell you see a lot of activity immediately around the nucleus. The Ca^{2+} released from one of these perinuclear puffs enters the nucleus very quickly and you can actually see it traversing through the nucleus and popping out at the other end.