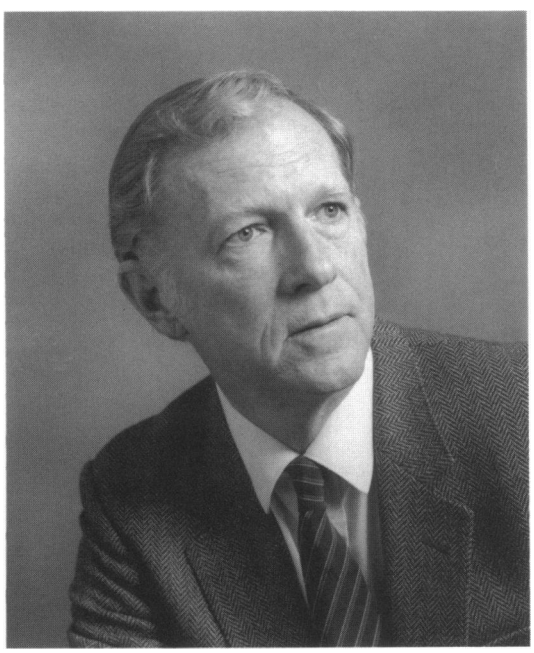

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Annual Review Prize Lecture

Elementary and global aspects of calcium signalling

Given at the Meeting of The Physiological Society held at the University of Edinburgh on 5 July 1996

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Calcium ions play a central role in the regulation of many forms of cellular activity. Control of muscle contraction or neurotransmitter release are obvious examples but calcium has been implicated in many other processes including synaptic plasticity, cell proliferation and cell death. In order to accommodate so many control functions, the mechanisms responsible for generating calcium signals are very diverse. Some are designed to give highly localized brief bursts of calcium whereas others produce longer-lasting global elevations of calcium which often appear as repetitive waves (Berridge & Dupont, 1994). Recent progress in our understanding of this complex spatiotemporal organization of calcium signalling has been facilitated by two major technological developments. Firstly, the tools of molecular biology have provided a detailed characterization of the structure and properties of calcium channels. Secondly, imaging technology has advanced to the point that the activity of individual channels can now be visualized in living cells. We are now in a unique position of being able to analyse how individual channels contribute to the development of calcium signals. When visualized in living cells, the activities of either single channels or small groups of channels may be considered as the elementary events of calcium signalling (Bootman & Berridge, 1995). In this article I shall explore how these elementary events are harnessed to create the highly localized or global calcium signals that regulate a wide range of cellular activities.

Basic aspects of calcium signalling

The cell has access to two sources of signal calcium: an infinite supply of external calcium and a more finite internal store sequestered within the endoplasmic/sarcoplasmic reticulum (ER/SR). The major channels responsible for regulating both entry and release are outlined in Fig. 1*A*.

Calcium entry channels

The three main entry channels have been classified on the basis of their regulatory mechanism – hence we have voltage-operated channels (VOCs), receptor-operated channels (ROCs) and store-operated channels (SOCs). These three channels have very different kinetic properties in that the VOCs and ROCs usually provide brief high intensity bursts whereas the SOCs provide a much smaller but sustained influx of calcium. While the operation of the VOCs and ROCs is reasonably well understood, the nature of the SOCs is still very much of a mystery (Bennett, Petersen & Cheek, 1995; Berridge, 1995; Clementi & Meldolesi, 1996). As first described by Putney (1986, 1990), the SOCs are regulated by the state of filling of the stores through a mechanism that has become known as capacitative calcium entry. When the stores are full there is no entry but as soon as they empty they begin to activate entry across the plasma membrane (Fig. 1*B*). The operation of these SOCs can be demonstrated in *Xenopus* oocytes following the emptying of the internal stores by treating the cells with lysophosphatidic acid (LPA) to stimulate the production of inositol 1,4,5-trisphosphate ($InsP_3$), by injecting $InsP_3$ directly or by using thapsigargin to inhibit the calcium pumps (Yao & Parker, 1993; Lupu-Meiri, Beit-Or, Christensen & Oron, 1993; Petersen & Berridge, 1994). Since these oocytes have calcium-activated chloride channels, the influx of calcium can be monitored by measuring the change in current when pulses of calcium are added to oocytes perfused with a calcium-free medium (Petersen & Berridge, 1994). Prior to agonist stimulation there was no response, but after depletion of the stores with LPA there was a gradual onset of capacitative calcium entry. The SOCs are particularly sensitive to calcium, which appears to have a biphasic effect in that it activates at low levels but becomes inhibitory at higher levels (Berridge, 1995).

A major unsolved problem concerns the nature of the mechanism that couples store emptying to channel opening (Bennett *et al.* 1995; Berridge, 1995). One suggestion is that the empty store generates a calcium influx factor (CIF) which then diffuses to the membrane to open the SOCs (Parekh, Terlau & Stühmer, 1993; Randriamampita & Tsien, 1993; Davies & Hallett, 1995). A putative CIF has been isolated but its exact structure remains to be determined (Randriamampita & Tsien, 1993; Thomas & Hanley, 1995). An alternative conformational coupling model considers that information is transferred more directly through the large cytoplasmic head of the $InsP_3$ receptor

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(InsP_3R; Fig. 2*A*) (Irvine, 1990; Berridge, 1990, 1995). This conformational coupling model is directly analogous to the model that has been proposed to describe depolarization-induced calcium release in skeletal muscle (Fig. 2*B*). In the latter case, depolarization induces the dihydropyridine receptors (DHPRs) to undergo a conformational change which is transferred to the type 1 ryanodine receptor (RyR1; thick arrows in Fig. 2*B*) resulting in a release of calcium from the SR. For capacitative calcium entry, information flows in the opposite direction – as a result of store emptying the InsP_3R (most probably the type 3 isoform; see Berridge, 1995) undergoes a conformational change that is transmitted to the SOCs in the plasma membrane to induce the influx of external calcium (Fig. 2*A*).

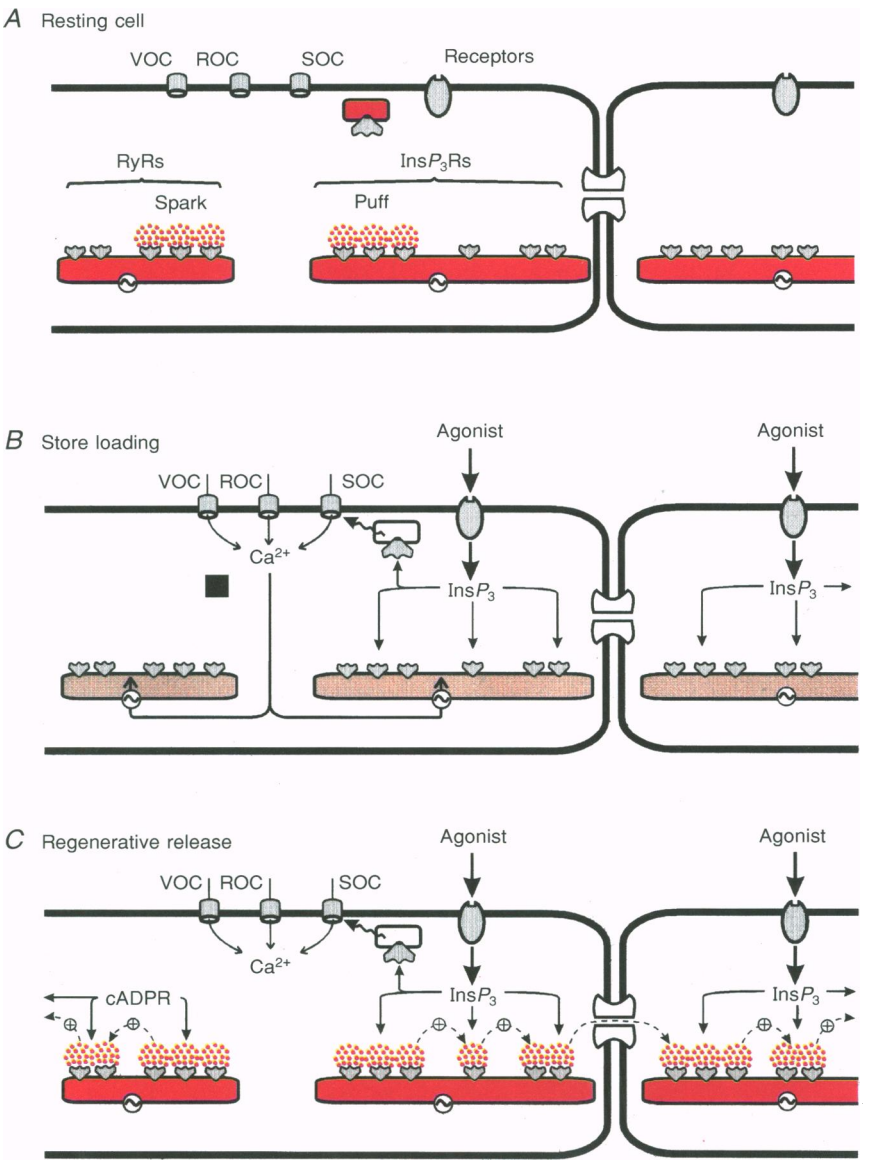

Figure 1. Functional organization of calcium channels

The main channels in the plasma membrane are the voltage-operated channels (VOC), the receptor-operated channels (ROC) and the store-operated channels (SOC). Release of calcium from the internal stores is mediated by either inositol 1,4,5-trisphosphate receptors (InsP_3Rs) or ryanodine receptors (RyRs). *A*, resting cells sometimes display the spontaneous release from the intracellular receptors to give sparks or puffs. *B*, loading of the internal stores by entry of external calcium. *C*, regenerative release through a process of calcium-induced calcium release (dashed arrows) to set up calcium waves that can pass from cell to cell through the gap junctions. Second messengers such as cyclic ADP ribose (cADPR) or InsP_3 enhance the calcium sensitivity of the RyRs and InsP_3Rs, respectively, thus converting the cytoplasm into an excitable medium capable of such regenerative activity.

For the conformational coupling model to work, a portion of the ER must lie within 10 nm of the plasma membrane to permit the InsP_3Rs to contact the SOCs. Such close juxtaposition of the ER to the cell surface has been described in *Xenopus* oocytes (Gardiner & Grey, 1983) and in *Drosophila* photoreceptors (Walz, 1982). The latter is particularly interesting because capacitative calcium entry has been implicated in the response of these photoreceptors to light (Hardie & Minke, 1993; Selinger, Doza & Minke, 1993). It is reasonable to speculate, therefore, that the entry channels in these *Drosophila* photoreceptors, i.e. the channels encoded by the transient receptor potential (*trp*) and the *trp*-like genes, may be homologous to the vertebrate SOCs. Indeed, homologues to *trp* have been described in *Xenopus* (Petersen, Berridge, Borgese & Bennett, 1995), mouse (Petersen *et al.* 1995; Zhu *et al.* 1996) and man (Wes, Chevesich, Jeromin, Rosenberg, Stetten & Montell, 1995; Zitt *et al.* 1996; Zhu *et al.* 1996). Inward currents that can be activated by store depletion have been obtained in cells following transfection with either *Drosophila trp* (Vaca, Sinkins, Hu, Kunze & Schilling, 1994; Petersen *et al.* 1995) or the mammalian homologues of *trp* (Zhu *et al.* 1996; Zitt *et al.* 1996). Identification of these SOCs and characterization of their function is of major importance because these channels provide the constant influx of calcium necessary to load up the internal stores (Fig. 1*B*) in order to sustain the repetitive spiking and waves found in a large number of non-excitable cells.

Intracellular calcium channels

The two intracellular channels responsible for releasing calcium from the internal stores are the InsP_3Rs and the ryanodine receptors (RyRs) (Fig. 1*A*). There are three RyRs and at least four InsP_3Rs. These two receptor families appear to have evolved from a common ancestor because they share many structural and physiological similarities (Berridge, 1993; Taylor & Traynor, 1995). Each receptor has membrane-spanning regions in their C-terminal domain, which serve to embed them in the ER/SR and also function as the calcium channel. The very large N-terminal region forms a bulbous head that projects into the cytoplasm and integrates the various signals that control channel opening and closing. Of particular significance with regard to the spatiotemporal aspects of signalling is that both receptor families display the process of calcium-induced calcium release (CICR), which accounts for their ability to generate calcium spikes and calcium waves. This calcium sensitivity enables the calcium released from one receptor to excite its neighbours thereby igniting a regenerative wave capable of sweeping through the cytoplasm (Fig. 1*C*). The cytoplasm may be considered as an 'excitable medium' (Lechleiter & Clapham, 1992). At rest, it is inexcitable but, upon stimulation, the calcium sensitivity of the InsP_3R and RyR begins to rise, through the action of second messengers such as InsP_3 and cyclic ADP ribose (cADPR), respectively, thus converting the cytoplasm into the excitable medium capable of generating repetitive calcium waves. This concept of calcium excitability has been incorporated into a mathematical model that adequately accounts for InsP_3-induced calcium oscillations (Li, Keizer, Stojilkovic & Rinzel, 1995)

In the case of InsP_3Rs, the major determinant of excitability is InsP_3 itself. The action of this second messenger may be somewhat more subtle than originally envisaged, when it was thought to act directly as a second messenger to release calcium (Streb, Irvine, Berridge & Schulz, 1983; Berridge &

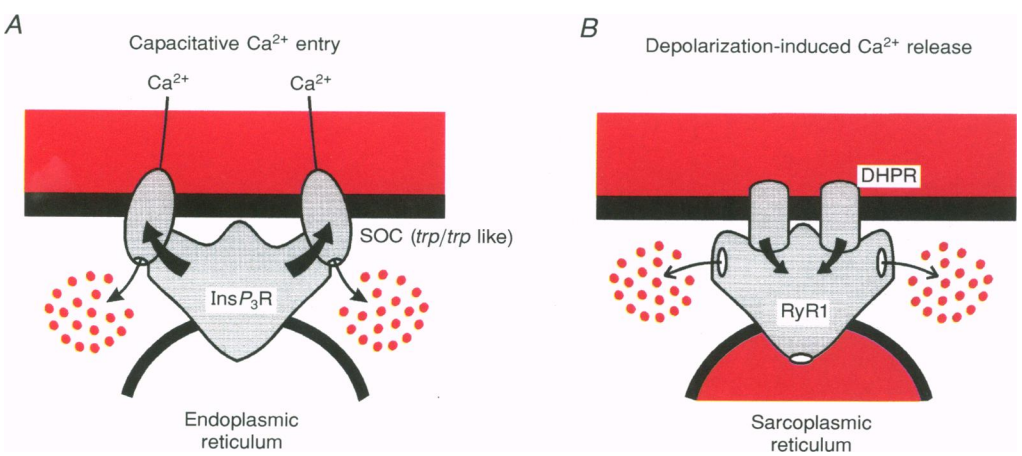

Figure 2. Conformational coupling mechanisms for transmitting information between the plasma membrane and the ER/SR calcium stores

A, a conformational coupling model of capacitative calcium entry. The InsP_3R undergoes a conformational change which then opens SOCs to gate the entry of external calcium. *B*, depolarization-induced release of calcium by the type 1 RyR (RyR1) in the SR. Depolarization is detected by dihydropyridine receptors (DHPR), which then induce a conformational change in the RyR1s resulting in the efflux of stored calcium. The function of this DHPR–RyR1 couple as a component of the elementary unit of calcium release in skeletal muscle is described later (see Fig. 5*A*).

Irvine, 1984). Such a mechanism may exist, especially during strong stimulation, when large surges of InsP_3 are produced. At normal physiological stimulation levels, however, the increase in InsP_3 may be insufficient to release calcium directly but it may set the stage for spontaneous calcium oscillations by sensitizing the InsP_3Rs (Fig. 1*C*). What is important, therefore, is that the InsP_3R is under dual agonist control in that opening depends on the presence of both InsP_3 and calcium (Fig. 1*C*). A beautiful example of this dual agonist control was described in *Xenopus* oocytes where the cell was divided into excitable and inexcitable regions by releasing InsP_3 into one half of the cell only (Yao & Parker, 1992). A regenerative calcium wave was found to propagate through the excitable half but failed to invade the remaining half where the InsP_3 level was low. The dual agonist concept appears to apply equally as well to the RyRs in that the putative calcium-mobilizing second messenger cADPR seems to act by enhancing the calcium sensitivity of the RyRs (Fig. 1*C*) (Lee, 1993).

In addition to these cytosolic regulators of release, there are indications that the level of calcium within the lumen of the ER/SR might be capable of adjusting the sensitivity of both the InsP_3Rs (Missiaen, Taylor & Berridge, 1992; Parys, Missiaen, De Smedt & Casteels, 1993; Horne & Meyer, 1995; Tanimura & Turner, 1996) and the RyRs (Donoso, Prieto & Hidalgo, 1995; Lukyanenko, Györke & Györke, 1996). Overloading cardiac myocytes results in a marked increase in the excitability of the RyRs, which is manifested by the appearance of regenerative calcium waves (Cheng, Lederer & Cannell, 1993; Cheng, Lederer, Lederer & Cannell, 1996*a*). Just how lumenal calcium might influence these receptors is still a matter for debate as there are no obvious calcium-binding domains on those segments of the receptors that face the lumen. One possibility is that the lumenal calcium may leak out and act through the cytosolic binding sites (Taylor & Traynor, 1995). Another possibility is that calcium acts indirectly through the lumenal buffers such as calsequestrin and calreticulin (Ikemoto, Antoniu, Kang, Meszaros & Ronjat, 1991; Kawasaki & Kasai, 1994). Attempts to modify calcium signalling by altering the content of these buffers have been equivocal. Increasing the calreticulin level in HeLa cells had little effect on agonist-induced responses (Bastianutto *et al.* 1995). On the other hand, reducing the level of calreticulin in neuroblastoma cells decreased bradykinin-induced signalling (Liu, Fine, Simons & Johnson, 1994). The calcium waves in *Xenopus* oocytes were inhibited by expressing calreticulin through an effect which seemed to be independent of its calcium storage property (Camacho & Lechleiter, 1995). Whatever the mechanism turns out to be, it seems that loading of the ER/SR does contribute to the increase in receptor sensitivity of both the InsP_3Rs and RyRs.

Quantal calcium release and graded responses

Given that InsP_3Rs and RyRs are excitable, i.e. they activate each other through the process of CICR, regenerative calcium signals should be all-or-none. However, there are numerous examples of release from both InsP_3Rs and RyRs being quantal (Bootman, 1994). This quantal phenomenon is best

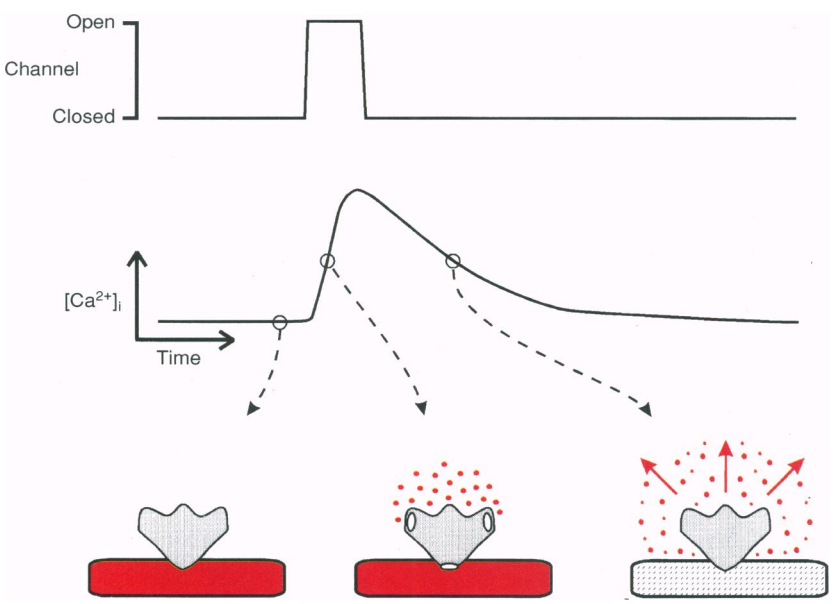

Figure 3. Schematic representation of a typical elementary calcium signalling event

Although most of the events recorded so far probably result from the co-ordinated opening of a small group of receptors, only a single channel has been depicted in this diagram for simplicity. The channel opens for a brief period, as shown in the top trace, resulting in the rapid release of calcium ($[Ca^{2+}]_i$, intracellular calcium concentration). Once the channel closes, the microdomain of calcium disperses by free diffusion. See Table 1 for a summary of the distribution and properties of some typical elementary events.

Table 1. Summary of the distribution and properties of some typical elementary events (see Fig. 3)

Elementary event	Tissue	Calcium channel	Duration at half-maximum	Reference
Puff	*Xenopus* oocyte	InsP_3R	250 ms	Yao *et al.* (1995)
Bump	*Limulus* photoreceptor	InsP_3R	50 ms	Wong, Knight & Dodge (1982)
Spark	Skeletal muscle	RyR1	26 ms	Klein *et al.* (1996)
Spark	Cardiac muscle	RyR2	40 ms	Cheng *et al.* (1993)
Spark	Smooth muscle	RyR(?)	35 ms	Nelson *et al.* (1995)
STOC	Smooth muscle	RyR(?)	65 ms	Benham & Bolton (1986)
SMOC	Sympathetic neuron	RyR(?)	30 ms	Marrion & Adams (1992)
STIC	Smooth muscle	RyR(?)	90 ms	Hogg, Wang, Helliwell & Large (1993)
Bump	*Drosophila* photoreceptor	*trp*	30 ms	Hardie (1991)
QED	Squid giant synapse	VOC	500 μs	Sugimori *et al.* (1994)

Duration at half-maximum, the width of the calcium transient at the point where its concentration is 50% of maximum. Abbreviations: STOC, spontaneous transient outward current; SMOC, spontaneous miniature outward current; STIC, spontaneous transient inward current; QED, quantum emission domain; InsP_3R, inositol 1,4,5-trisphosphate receptor; RyR1, type 1 ryanodine receptor; RyR2, type 2 ryanodine receptor; VOC, voltage-operated channel.

seen in permeabilized cells in which the stores appear to have variable sensitivities such that more and more of the stores are activated as the level of InsP_3 is increased. Quantal calcium release can also be demonstrated in single intact cells which release calcium through either InsP_3Rs (Bootman, Berridge & Taylor, 1992; Parker & Ivorra, 1993; Bootman, 1994) or RyRs (Cheek, Moreton, Berridge, Stauderman, Murawsky & Bootman, 1993). This quantization of the internal release channels seems to be responsible for the graded calcium signals that have been described in certain cells such as HeLa cells (Bootman, Cheek, Moreton, Bennett & Berridge, 1994; Bootman, 1996) and cardiac cells (Isenberg & Han, 1994). How is it that cells can generate graded responses when the elementary units are using a regenerative process which should give all-or-none responses? The solution to this paradox is that the calcium signalling system is composed of elementary events that function autonomously. Since they appear to have different sensitivities, the number available to contribute to a response varies with stimulus intensity (Bootman & Berridge, 1995). Attention is now focused on the composition and properties of these elementary events and how they are co-ordinated to give global responses.

Elementary and global events

With advances in imaging technology it has become feasible to visualize the elementary events which constitute calcium signalling. In most cases studied so far, the elementary events which have been recorded are probably not fundamental events, in the sense that they represent the opening and closing of single channels as will be discussed later, but are more likely to reflect localized groups of channels which are linked together as functional units during the development of a typical calcium signal. Such elementary events are providing new insights into the way in which calcium channels operate *in situ* to generate calcium signals. These elementary events, which have now been recorded in many different cell types (Table 1), are all characterized by a rapid rate of rise followed by a slower recovery period (Fig. 3). These kinetics are consistent with the rapid 'on' and 'off' events seen in single channel recordings. As the channel opens, calcium diffuses out rapidly to create a plume which then dissipates more slowly by diffusion once the channel closes (Fig. 3). As described earlier, the opening and closing of the release channels (InsP_3R and RyR) is strongly influenced by the positive and negative feedback effects of calcium. The latter will limit the amount of calcium being released during each elementary event and will thus determine the amplitude of the global event because it is the summation of these elementary events which gives rise to the whole-cell responses of spikes and waves. Such elementary events have been described for both calcium entry and release channels and have a variety of names often reflecting their spatiotemporal properties, e.g. sparks, puffs, bumps and quantum emission domains (QED). In some cases, the existence of these events has been inferred on the basis of brief calcium-dependent current transients such as the spontaneous transient outward currents (STOCs) in smooth muscle (Benham & Bolton, 1986; Zholos, Komori, Ohashi & Bolton, 1994) or the spontaneous miniature outward currents (SMOCs) in neurons (Marrion & Adams, 1992).

The elementary events outlined in Fig. 3 and Table 1 have at least three basic functions. These spontaneous events can contribute to the resting level of calcium. They can provide highly localized pulses of calcium to regulate certain physiological processes such as exocytosis or the activation of ion channels, or they can contribute to the development

of a global elevation of calcium to activate more distant effector systems (Fig. 4). To better understand these different functions, it is necessary to describe how the elementary events of different calcium channels contribute to calcium signalling in some specific examples.

Contribution of elementary events to the resting level of calcium

The resting level of calcium in the cytoplasm results from a balance between the basal rates of calcium input and removal. Since elementary events, such as sparks and puffs, release a bolus of calcium to the cytoplasm (Fig. 4*A*) they will contribute to the resting or basal level of calcium. Studies on smooth muscle have revealed that elementary events, represented by STOCs, can have a marked influence on determining this resting level of calcium. For example, inhibition of STOC activity by caffeine or ryanodine was found to decrease the background potassium current, consistent with a decrease in the resting level of calcium (Désilets, Driska & Baumgarten, 1989). More direct measurements have revealed that the intracellular level of calcium fluctuates significantly depending upon STOC frequency (Ganitkevich & Isenberg, 1996). Evidence that such elementary events can contribute to the resting level of calcium has also been obtained in HeLa cells (Bootman & Berridge, 1996) and in *Xenopus* oocytes (Parker & Yao, 1996; Parker, Choi & Yao, 1996*a*). In conclusion, in resting cells or in cells receiving low levels of stimuli, the small bolus of calcium released to the cytoplasm during each elementary event can exert a marked effect on the resting level of calcium. Such elevations in calcium will enhance the excitability of the intracellular receptors and could thus contribute to the onset of global calcium signals.

Localized signalling functions of elementary events

Elementary events generate a highly concentrated localized burst of calcium that can perform very specific signalling functions. A classical example is the triggering of exocytosis by a brief pulse of calcium entering through VOCs. These elementary events, detected as QEDs (Fig. 3), last for about 500 μs during which the calcium concentration increases

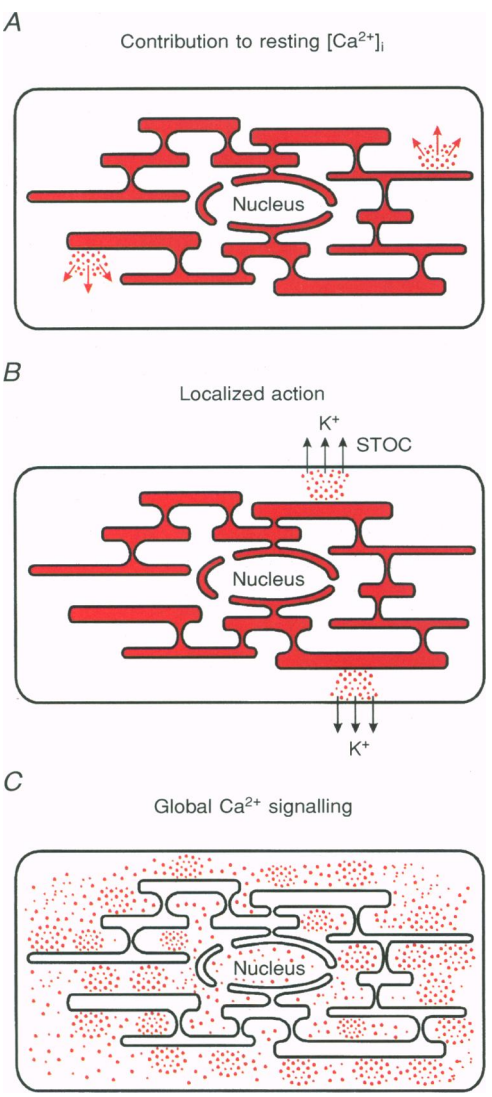

Figure 4. Functions of elementary events

A, the resting level of calcium may be modified by spontaneous elementary events releasing brief bursts of calcium into the cytoplasm. *B*, elementary events can have localized effects such as the activation of potassium channels to produce the spontaneous transient outward currents (STOCs) that have been recorded from smooth muscle cells. *C*, when elementary events are co-ordinated they give rise to a global calcium signal throughout the cytoplasm and nucleus.

from 100 nM up to about 200 μM (Llinás, Sugimori & Silver, 1992; Sugimori, Lang, Silver & Llinás, 1994). Since the VOCs associate with synaptic vesicle proteins such as syntaxin and the 25 kDa synaptosome-associated protein (SNAP25) (Sheng, Rettig, Cook & Catterall, 1996), QEDs will be positioned in the immediate vicinity of the docked vesicle and can thus trigger exocytosis with a minimum of delay.

Regulation of mitochondrial metabolism is another example of a localized signalling function for elementary events. There is evidence that mitochondria respond to the high levels of calcium that exist in the vicinity of the release sites (Rizzuto, Brini, Murgia & Pozzan, 1993; Lawrie, Rizzuto, Pozzan & Simpson, 1996). The affinity of the calcium uptake mechanism is too low for mitochondria to detect the global elevations in calcium concentration (usually $\leqslant 1$ μM), but they can respond to the much larger calcium signals near the elementary events and this uptake of calcium results in an increase in respiratory metabolism (Hajnoczky, Robb-Gaspers, Seitz & Thomas, 1995). An interesting aspect of this mechanism is that mitochondrial metabolism is much more sensitive to oscillating calcium signals than to maintained signals. The mitochondria are tuned to calcium oscillations because they detect the high levels of calcium associated with the burst of elementary events that make up the rising phase of each cycle (Hajnoczky *et al.* 1995). Whether or not mitochondria tune in to these elementary events will largely depend on their location relative to the calcium channels (Lawrie *et al.* 1996). In some cells, the mitochondria respond to calcium entry across the plasma membrane whereas in other cells they are sensitive to the release of internal calcium.

Another important localized action of elementary events is to activate ion channels as has been described in smooth muscle (Benham & Bolton, 1986; Bolton & Lim, 1989; Nelson *et al.* 1995). Subsarcolemmal calcium sparks stimulate localized potassium channels (Fig. 4*B*) to give STOCs which can be dissociated from the global elevation of calcium (Stehno-Bittel & Sturek, 1992; Ganitkevich & Isenberg, 1996). Indeed, the STOCs in arterial smooth muscle may perform a specific function by stimulating the efflux of potassium; the STOCs hyperpolarize the membrane thus leading to relaxation (Nelson *et al.* 1995). Small bursts of calcium near the membrane lead to relaxation whereas the more diffuse global elevation stimulates contraction. In summary, elementary events not only contribute to global calcium signals but they may also have very precise localized signalling functions such as the relaxation of arterial smooth muscle or the exocytosis of secretory vesicles.

Global calcium signalling

The calcium signals that are familiar to most physiologists are the global calcium signals arising from the synchronized release of calcium from a large proportion of the intracellular channels (Fig. 4*C*). Because of the potent negative feedback effect of calcium, each channel is open for a brief period, which means that global responses depend upon synchronization of the elementary events described earlier. Cells appear to have evolved two mechanisms to co-ordinate these elementary events (Bootman & Berridge, 1995). Firstly, channel opening can be evoked nearly simultaneously (i.e. within milliseconds) by being tightly coupled to an action potential in the plasma membrane as occurs in skeletal, cardiac, and some smooth muscle cells. Secondly, the channels co-ordinate their own activity through the regenerative process of CICR. This process, which is present in non-excitable cells, is much slower because the synchronization signal is calcium itself diffusing from one channel to the next, usually in the form of a calcium wave taking several seconds to traverse a typical cell.

Evoked calcium release from skeletal muscle ryanodine receptors (RyR1s)

The SR of skeletal muscle contains the type 1 RyR which responds to T-tubule depolarization by releasing the calcium responsible for triggering contraction. Because contraction occurs rapidly and repeatedly, the calcium signalling system is designed to deliver a near simultaneous burst of calcium throughout the large muscle fibre. The synchronization of release is achieved by having the activity of the RyRs tightly coupled to the action potential that sweeps through the T-tubules. The depolarization of the T-tubule is sensed by the DHPRs which then activate the RyR1s through a direct protein–protein interaction as described earlier (Fig. 2*B*) (Meissner & Lu, 1995). Indeed, it was this conformational coupling model, first proposed by Schneider & Chandler (1973), which was the inspiration for the calcium entry model described earlier (Fig. 2*A*). In both cases, the large cytoplasmic heads of the calcium channels are used for the transfer of information between the two membranes (i.e. the plasma membrane and the membrane of the calcium store). It has been possible to obtain direct evidence that these two proteins interact with each other by using immunoprecipitation techniques with specific antibodies against DHPRs or RyR1s (Marty *et al.* 1994). The conformational change that occurs in the RyR has been detected using a fluorescence tag (Yano, El Hayek & Ikemoto, 1995). Not only does the DHPR open the RyR1 upon depolarization but it is also responsible for closing the release channel upon repolarization (Suda, 1995). The fact that calcium release is under bidirectional control through both depolarization and repolarization indicates a tight coupling between the DHPR and the RyR. This reciprocal interaction between the two channels has also been highlighted by showing that DHPR function in dyspedic mice myotubes can be restored by expressing RyR1 (Nakai, Dirksen, Nguyen, Pessah, Beam & Allen, 1996). It is proposed that, in addition to the DHPR being able to influence the RyR1, a retrograde signal can travel in the opposite direction. Such studies emphasize the tight coupling between these two proteins. Since the RyRs outnumber the DHPRs, a proportion of the former are not coupled and must either be silent or, as illustrated in Fig. 5*A*, they might be recruited by a separate process of CICR. The elementary unit therefore is a DHPR–RyR1

couple together with some neighbouring free RyR1s (Fig. 5*A*). Image analysis of events within each triad has begun to reveal new principles concerning the way in which this elementary unit contributes to the global calcium signal during excitation–contraction (E–C) coupling (Tsugorka, Rios & Blatter, 1995; Klein, Cheng, Santana, Jiang, Lederer & Schneider, 1996; Schneider & Klein, 1996).

In resting muscle, there are spontaneous sparks which may represent a fundamental event in that they seem to result from the opening of a single RyR1. The frequency of these spontaneous sparks was enhanced by raising the resting level of calcium or by adding caffeine and may represent the activity of those RyRs which are not directly coupled to the DHPRs (Klein *et al.* 1996). The spark frequency was greatly enhanced by membrane depolarization. The nature of these evoked events are best seen at low levels of membrane depolarization when the incidence of sparks is increased as individual DHPR–RyR1 couples begin to be active (Tsugorka *et al.* 1995; Klein *et al.* 1996). Unlike the spontaneous sparks which have a unitary amplitude, these evoked sparks reveal multiple amplitudes which appear to grow in quantal steps suggesting that some fundamental event can recruit neighbouring receptors, which constitutes the elementary event (Tsugorka *et al.* 1995; Klein *et al.* 1996). The specific proposal is that membrane depolarization activates the DHPR–RyR complexes to give the initial burst of calcium which then recruits uncoupled RyR1s (i.e. those not bound to DHPR) through a process of CICR (Fig. 5*A*) (Klein *et al.* 1996). The global calcium signal in skeletal muscle seems to be derived from both depolarization- and ligand-activated events.

Evoked calcium release from cardiac muscle ryanodine receptors (RyR2s)

In cardiac muscle, contraction is triggered by release of calcium from ryanodine type 2 receptors (RyR2s) located in the SR. As for skeletal muscle, release is driven by membrane depolarization again using DHPRs as voltage sensors, but in cardiac muscle there is no physical contact between the two elements (Fig. 5*B*). Instead, coupling is mediated by the process of CICR in that the DHPR opens in response to depolarization to give a brief pulse of trigger calcium which then activates the underlying RyRs. Previous physiological measurements had already led to the concept of local control in that the release mechanism appeared to be divided up into independent units in which a DHPR communicated directly with a small group of RyRs (Stern, 1992; Wier, Egan,

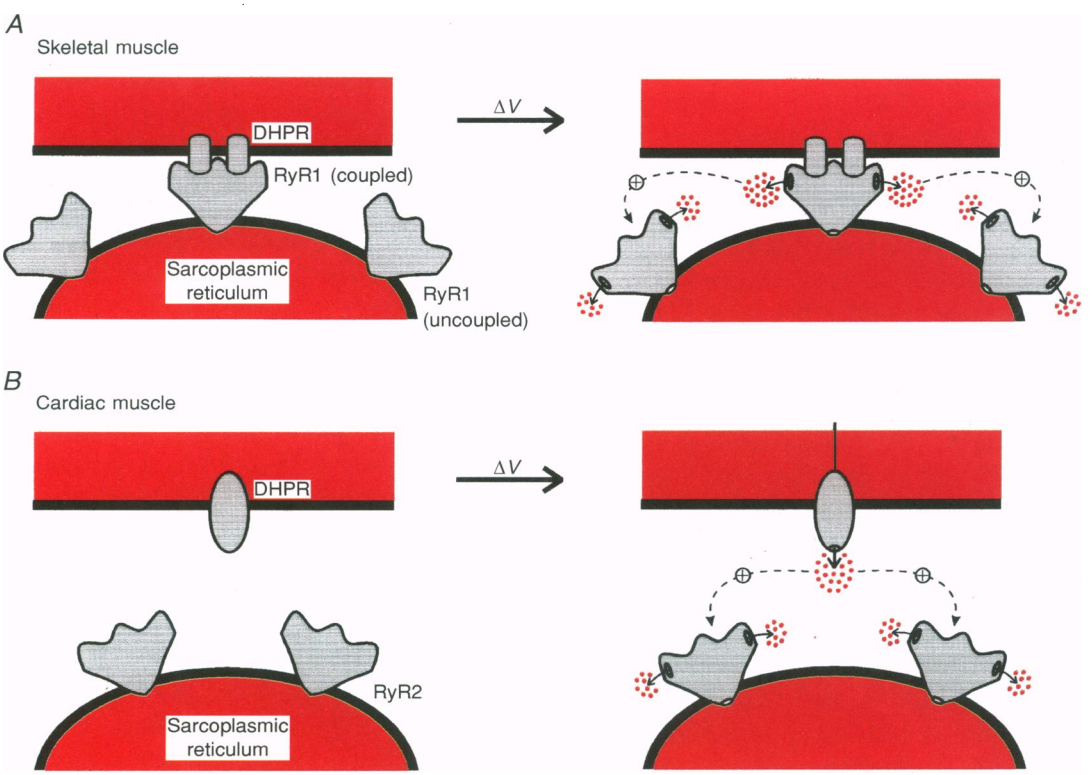

Figure 5. Elementary units of calcium signalling in muscle cells

A, skeletal muscle SR has RyR1s some of which are coupled to the DHPRs. Upon depolarization (ΔV) the DHPRs undergo a conformational change to trigger the coupled RyR1s to release calcium which can be amplified further by CICR when calcium acts as an agonist to stimulate the uncoupled RyR1s. *B*, the cardiac muscle elementary unit consists of a DHPR closely associated with approximately four RyR2s (only two shown for simplicity). Upon depolarization, the DHPR opens to provide a pulse of trigger calcium which then activates the RyR2s.

López-López & Balke, 1994; Isenberg & Han, 1994). Stern (1992) introduced the idea of a 'calcium synapse' to draw attention to the idea of calcium acting as a messenger to couple the DHPR to the RyRs. The spatial and temporal summation of these independent units generates the global calcium signals that develop during normal E–C coupling (López-López, Shacklock, Balke & Wier, 1994, 1995; Cannell, Cheng & Lederer, 1994, 1995). The reason why they can be considered as independent release units or elementary events is because the low calcium sensitivity of the RyRs means that neighbouring units or synapses are functionally uncoupled from each other (Niggli & Lederer, 1990). The beauty of this local control concept is that it can reconcile the paradox mentioned earlier about calcium signals being graded even though they are being generated through the regenerative process of CICR. This paradox is particularly evident in heart where the amplitude of calcium signals is graded by the recruitment of elementary release units depending on the number of DHPRs activated at each membrane voltage (Isenberg & Han, 1994). Furthermore, calcium release initiated by membrane depolarization can be rapidly terminated by switching off the current flowing through the DHPRs (Wier *et al.* 1994). Because of the stochastic property of the L-channels, i.e. they open very briefly (0·2 ms) and somewhat infrequently (open probability, 0·04), not all the available units will be activated at any given time, which explains how the activation process can be terminated so rapidly just by shutting off the DHPRs. The single most important feature to emerge from all these studies is that the global elevation of calcium in response to membrane depolarization is achieved by the recruitment of elementary units based on an interaction between the DHPRs and RyR2s.

Confocal microscopy has begun to characterize the properties of these elementary units. Resting cardiac cells display calcium sparks (Cheng *et al.* 1993) which have all the characteristics of a typical elementary event as described earlier (Fig. 3). Within the 1 μm confocal image section, these sparks occurred at a low frequency of approximately one per second. The increase in calcium reached a peak in 10 ms and then showed the characteristic slow decay (half-time of the decay of the calcium spark, $t_{½}$, 20 ms). Based on the amount of calcium being released, it was calculated that these sparks represent the opening of either a single or a small group of RyR2s (Cheng *et al.* 1993). Subsequent studies have helped to confirm that these sparks are the elementary units of calcium signalling in cardiac calls (López-López *et al.* 1994, 1995; Cannell *et al.* 1995; Cheng *et al.* 1996*b*). In previous studies, the muscle was scanned longitudinally and this led to the concept that sparks were autonomous events spaced at intervals of 1·8 μm, which corresponded to the position of the T-tubules (Shacklock, Wier & Balke, 1995; Cheng *et al.* 1996*b*). However, when scanned in the transverse direction (i.e. along the plane of the Z-line) the sparks occurred closer to each other and there was evidence for a localized coupling between spark sites (Parker, Zang & Wier, 1996*b*). The concept of autonomy can be retained because the coupling was confined to a few neighbouring spark sites and failed to spread throughout the Z-disc region.

In keeping with the calcium synapse concept, the frequency of such sparks is greatly enhanced as the membrane is progressively depolarized (Cannell *et al.* 1995). Both the spontaneous and evoked sparks had very similar amplitudes and there appeared to be a tight coupling between the influx of trigger calcium entering through the DHPR and the incidence of the calcium sparks (Cannell *et al.* 1995; López-López *et al.* 1995; Santana, Cheng, Gomez, Cannell & Lederer, 1996). It seems that the elementary event comprises a single DHPR regulating the activity of approximately four RyRs (Cannell *et al.* 1994). Lipp & Niggli (1996) have suggested that this elementary event, comprising a DHPR and a tightly coupled group of RyR2s (Fig. 5*B*), might be further subdivided into 'calcium quarks', which would be the fundamental event as it is thought to result from the opening of a single channel. Such quarks have not been observed directly but have been inferred from experimental manipulations involving calcium release induced by photolytic pulses of calcium. This quark concept emphasizes the hierarchical organization of calcium signalling (Lipp & Niggli, 1996). The quarks (fundamental events) are recruited to give sparks (elementary events) which, in turn, are recruited to give the cellular calcium transients (global events). As will be described later, such a hierarchical organization applies equally as well to calcium signalling in non-excitable cells (Bootman, 1996; Parker *et al.* 1996*a*; Bootman, Niggli, Berridge & Lipp, 1997).

In the heart, the basic units that operate under normal physiological conditions following membrane depolarization are the calcium sparks. An analysis of the events that occur during such sparks can help to explain a number of aspects of E–C coupling. For example, the degree to which the RyRs amplify the trigger calcium entering through the DHPRs constitutes the amplification or gain of this transducing mechanism. Cannell *et al.* (1995) consider that this gain has both digital and analog components. Analog gain arises from the RyRs having a greater conductance than the DHPR whereas digital gain depends on the fact that the RyRs remain open for longer. The combination of these two components means that a small flux through the DHPR is amplified by a much larger efflux from the RyRs. The summation of such high gain, all-or-none elementary release units generates the global elevation necessary to contract cardiac cells during each heart beat.

Evoked and spontaneous calcium release in smooth muscle

The enormous variability that exists between different smooth muscle cells makes it difficult to draw general conclusions concerning their calcium signalling mechanisms. They use both depolarization-dependent and agonist-dependent mechanisms (Somlyo & Somlyo, 1994). Some muscles, such as those in arteries and veins, display a mechanism similar to that in cardiac cells where entry of

calcium through VOCs is further amplified by CICR from internal stores (Ganitkevich & Isenberg, 1992, 1995; Grégoire, Loirand & Pacaud, 1993). In contrast to cardiac cells, however, these smooth muscle cells operate a low gain mechanism in that the entry of external calcium contributes a larger proportion of the global calcium signal. In addition to this depolarization-dependent mechanism, many smooth muscle cells also employ an agonist-dependent process which uses $InsP_3$ to release calcium from internal stores (Somlyo & Somlyo, 1994). Calcium signals are generated by both $InsP_3$Rs and RyRs and there are indications that these two release channels might co-operate with each other in some smooth muscle cells.

As for skeletal and cardiac muscle, calcium sparks have been recorded and these have begun to reveal some new principles concerning the contribution of these elementary events to smooth muscle contraction. Some of the first indications of such elementary events were the STOCs recorded in visceral and vascular smooth muscle (Benham & Bolton, 1986; Bolton & Lim, 1989; Désilets *et al.* 1989). These STOCs had the characteristic features of a typical elementary event (Fig. 3). A localized release of calcium from an internal store opened 75–100 calcium-sensitive potassium channels in the plasma membrane to give a STOC (Fig. 4*B*) (Benham & Bolton, 1986). Confocal microscopy has now visualized the calcium signal mediating these STOCs as localized sparks occurring near the membrane of smooth muscle cells of cerebral arteries (Nelson *et al.* 1995) and rat portal vein (Mironneau, Arnaudeau, Macrez-Lepretre & Boittin, 1996). The role of such STOCs in mediating relaxation of some smooth muscle cells was described earlier. Here we consider how STOCs might contribute to the global responses responsible for inducing contraction.

In order to generate the global calcium signals responsible for contraction there has to be a large increase in the frequency of such elementary events. As in skeletal and cardiac muscle, some smooth muscles such as guinea-pig coronary artery use membrane potential to achieve this acceleration as revealed by the large depolarization-induced increase in STOC frequency (Fig. 6) (Ganitkevich & Isenberg, 1995). It must be remembered, however, that the STOCs record sparks near the plasma membrane and may not necessarily reflect the sparks deeper within the cell responsible for global calcium signals. Therefore, the following discussion is based on the assumption that STOCs reflect the excitable state of all the intracellular release channels. The ability of membrane depolarization to enhance STOC frequency appears to be related to store loading because, after the stores were discharged with caffeine, depolarization caused little STOC activation and no change in global calcium. Note the complete cessation of spontaneous STOCs immediately following the caffeine response (Fig. 6). After 1 min, however, both STOCs and a global calcium signal began to reappear. Others have also noted that STOCs appear more frequently when stores are loaded (Stehno-Bittel & Sturek, 1992), whereas they were abolished when the stores were emptied by thapsigargin (Nelson *et al.* 1995) or by continuous treatment with high levels of caffeine (Désilets *et al.* 1989; Bolton & Lim, 1989; Stehno-Bittel & Sturek, 1992; Ganitkevich & Isenberg, 1995). A global elevation of calcium in these smooth muscle cells appears to be associated with an increase in STOC frequency. In some cases where a maintained depolarization

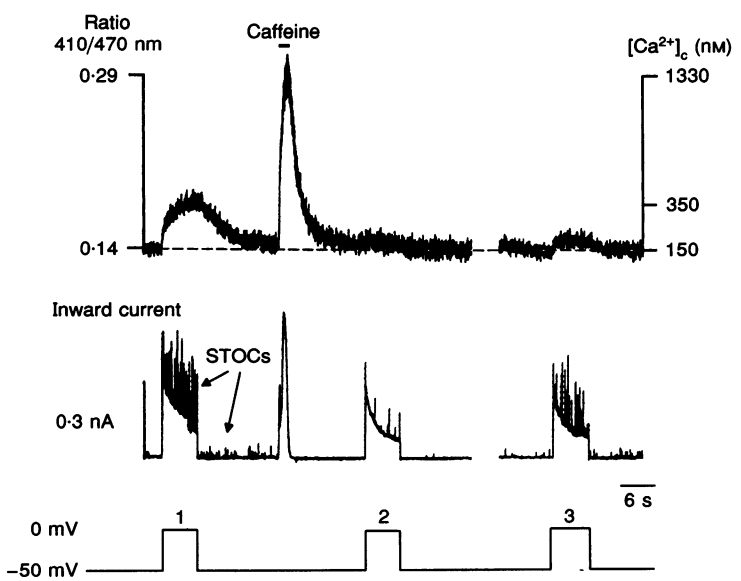

Figure 6. Depolarization- and caffeine-induced calcium release in guinea-pig coronary artery

The muscle was voltage clamped at −50 mV and then depolarized to 0 mV at three different times. Between the first and second depolarizations, the cell was treated with 10 mM caffeine for 2 s. Because of the difference in driving force for potassium efflux, the STOCs at −50 mV are smaller than those during the three periods of depolarization. Figure taken from Ganitkevich & Isenberg (1995). $[Ca^{2+}]_c$, cytosolic calcium concentration.

resulted in calcium oscillations, there was a large increase in STOC frequency during the rising phase of each transient (Ganitkevich & Isenberg, 1995). Also, it is clearly evident from Fig. 6 that the amplitude of the global response appears to be graded according to STOC frequency, suggesting that the tonic calcium signal is built up by the summation of elementary units.

In addition to membrane depolarization, STOC frequency can be influenced by a number of factors, most of which can be related to a change in the sensitivity of the intracellular receptors. For example, STOC frequency was accelerated by caffeine (Benham & Bolton, 1986; Bolton & Lim, 1989; Rusko, Wang & van Breemen, 1995; Ganitkevich & Isenberg, 1996), by stimulation with agonists such as carbachol (Bolton & Lim, 1989) or histamine (Désilets *et al.* 1989), or by repeated injections of InsP_3 (Zholos *et al.* 1994). During many of these responses there is often a marked acceleration of STOC frequency just before the onset of the regenerative global response. The way in which elementary events contribute to the development of the pacemaker elevation in calcium that precedes the onset of global calcium signals is explored further in the next section on spontaneous calcium spikes and waves.

Spontaneous calcium release – generation of spikes and waves in non-muscle cells

A characteristic feature of calcium signalling in many cells is its complex spatiotemporal organization. When cells are stimulated with agonist concentrations that lie within the physiological dose–response range, calcium signals invariably appear as repetitive calcium spikes that emerge spontaneously from a low resting level of calcium. Perhaps the most dramatic examples are the calcium spikes in hepatocytes that appear following stimulation with glycogen-mobilizing agents such as noradrenaline or vasopressin (Woods, Cuthbertson & Cobbold, 1986; Rooney, Sass & Thomas, 1990). As described for many other cell types, these hepatocyte spikes have a characteristic spatial organization in that they begin at a discrete initiation site and then spread through the cell as a regenerative wave (Rooney *et al.* 1990; Kawanishi *et al.* 1995). Other examples of such initiation sites have been described in pancreatic cells (Thorn, Lawrie, Smith, Gallacher & Petersen, 1993), smooth muscle cells (Iino, Yamazawa, Miyashita, Endo & Kasai, 1993; Mahoney, Slakey, Hepler & Gross, 1993), mammalian eggs (Miyazaki, Hashimoto, Yoshimoto, Kishimoto, Igusa & Hiramoto, 1986; Cheek, McGuinness, Vincent, Moreton, Berridge & Johnson, 1993), *Xenopus* oocytes (Parker & Yao, 1991), ascidian eggs (Speksnijder, Sardet & Jaffe, 1990; Speksnijder, 1992) and HeLa cells (Bootman & Berridge, 1996). The problem, therefore, is to explain the events responsible for the initiation and propagation of such spontaneous calcium spikes and waves.

A suitable starting point is to return to the concept of an excitable medium, which was introduced to describe the onset of calcium waves in *Xenopus* oocytes developed earlier (Lechleiter & Clapham, 1992). The spiral waves in *Xenopus* were found to initiate from discrete foci (Lechleiter, Girard, Peralta & Clapham, 1991; Lechleiter & Clapham, 1992). Previous studies had established that these foci seemed to have a much higher sensitivity to InsP_3 (Parker & Yao, 1991). Recent studies have begun to dissect out the elementary events which make up such foci and contribute to wave propagation (Yao, Choi & Parker, 1995; Parker & Yao, 1996; Parker *et al.* 1996*a*; Bootman *et al.* 1997). As is the case for sparks in muscle cells, the elementary events in *Xenopus* are best seen at very low levels of stimulation, which has revealed the existence of a hierarchical organization of calcium signalling (Fig. 7) (Bootman, 1996; Lipp & Niggli, 1996; Parker & Yao, 1996; Bootman *et al.* 1997). At low levels of InsP_3, which limits the number of InsP_3Rs that are active at any given time, the primary action of InsP_3 is to produce a small elevation of the resting level of calcium, which seems to result from the random opening of single InsP_3Rs to give so-called blips (Parker & Yao, 1996), which might be equivalent to the quarks in muscle (Lipp & Niggli, 1996). Such blips, which have an amplitude of 30 nm and a diameter of about 1·3 μm, have been visualized in HeLa cells responding to histamine (Bootman *et al.* 1997). When the concentration of InsP_3 was increased, the blips in *Xenopus* oocytes transformed into puffs (Fig. 7) which were somewhat larger but retained the characteristics of an elementary event described earlier (Fig. 3). These puffs, which resemble the sparks in muscle cells, result from the concerted activation of a local group of InsP_3Rs which were sufficiently excitable (i.e. they had bound InsP_3) to interact with each other to give a localized burst of calcium. It is proposed that such puffs are autonomous events that fail to spread because neighbouring groups of InsP_3Rs are not sufficiently excitable to respond to the calcium diffusing away from the puff (Parker & Yao, 1996; Parker *et al.* 1996*a*). When the sensitivity of these receptors was increased by further elevations in InsP_3 concentration, the puffs were found to expand, resulting in the appearance of regenerative calcium waves (Fig. 7).

The analysis of elementary events has thus begun to define the nature of wave initiation sites. In the case of HeLa cells, calcium puffs appear to function as a triggering event for the onset of a global wave (Bootman *et al.* 1997). The InsP_3Rs at these puff sites appear to be particularly sensitive and thus mark where elementary events are seen first. As the concentration of InsP_3 rises, first the blips and then the puffs provide the first indications of an increase in excitability which will extend throughout the cytoplasm when the InsP_3 concentration increases above a critical threshold level (Bootman *et al.* 1997). The InsP_3Rs that make up this excitable medium can set up global signals by communicating with each other using calcium as a messenger. The process begins at the initiation sites and is then perpetuated by a process of CICR as calcium diffuses from one receptor to the next (Fig. 7). The rate of calcium diffusion from one channel to the neighbouring release site

is the rate-limiting step that determines the rate of wave propagation (Wang & Thompson, 1995). When the cytosol is fully excitable, the calcium wave in HeLa cells progresses smoothly. At intermediate levels of excitability, however, the wave becomes saltatory and the basic regenerative units appear to be calcium puffs spaced 7·5 μm from each other (Bootman *et al.* 1997). Saltatory waves have also been visualized in those cells in which the release units are widely separated (Yagodin, Holtzclaw, Sheppard & Russell, 1994). The study of elementary events in *Xenopus* oocytes and HeLa cells has provided new insights concerning the concept of an excitable medium and has shown how the activities of individual InsP_3Rs are co-ordinated to initiate and propagate global calcium waves.

The concept of an excitable medium composed of calcium-releasing units with a hierarchical organization may well account for the spatial and temporal events of calcium signalling in many of the cells described earlier (Bootman, 1996). An example of agonist-induced increases in excitability has been described during the repetitive spikes induced by histamine in HeLa cells (Bootman & Berridge, 1996). The pacemaker elevation preceding each spike is characterized by random bursts of calcium whose frequency increases towards the onset of the regenerative phase of each spike. Similar brief bursts of calcium may contribute to the pacemaker ('foot') of the carbachol-induced calcium spike in smooth muscle cells (Iino *et al.* 1993). These brief bursts of calcium are very reminiscent of the calcium sparks in muscle or the puffs in *Xenopus* oocytes and may reflect the increase in excitability of the InsP_3Rs, which sets the stage for the onset of the regenerative response. What is interesting about these observations, however, which is not predicted by a mathematical treatment of such excitable media (Li *et al.* 1995), is that the InsP_3Rs undergo cycles of release long before the onset of the regenerative wave. Indeed, the appearance of such elementary events coincides with a progressive increase in the overall level of calcium and this may help to further enhance the excitability of all of the receptors to the point at which they can begin to be entrained to each other to give a global calcium signal. What emerges from this work on HeLa cells is that the build up to a spontaneous calcium spike is marked by a progressive increase in the excitability of the InsP_3Rs (Bootman & Berridge, 1996). As for the puffs in *Xenopus* oocytes, the receptors (or groups of receptors) function as autonomous units giving brief bursts of calcium independently of each other. As their excitability increases, however, this autonomy begins to break down such that they can begin to communicate with each other to generate a global calcium signal. It is not clear what determines the gradual increase in excitability during the pacemaker period but it may

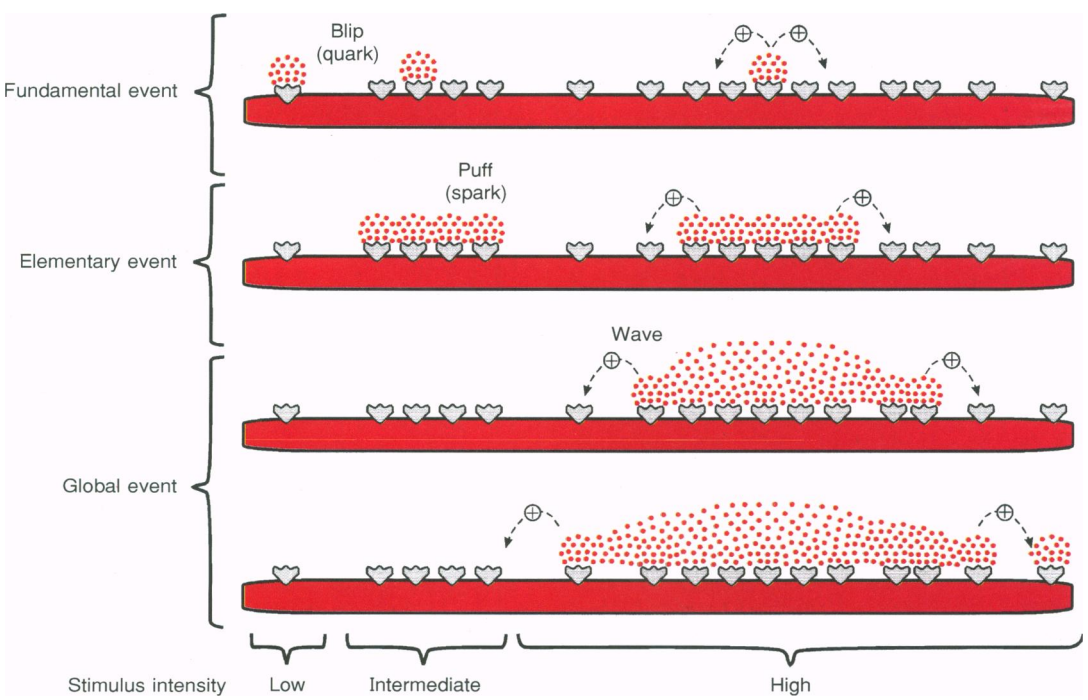

Figure 7. A hierarchical organization of intracellular calcium signalling

The stimulus intensity seems to determine which events are elicited. Fundamental events are the consequence of opening single intracellular channels to give blips (InsP_3Rs) or quarks (RyRs). Elementary events, represented by puffs and sparks, result from the concerted opening of small groups of InsP_3Rs or RyRs, respectively. These elementary events appear to be the basic building blocks of the global events which develop as a wave resulting from the progressive recruitment of neighbouring receptors through a process of CICR.

depend, at least in part, upon an influx of external calcium leading to an increase in store loading (Fig. 1*B*). In the case of *Xenopus* oocytes, treatments that enhance calcium influx accelerate spike frequency and also increase the rate of wave propagation (Girard & Clapham, 1993; Yao & Parker, 1994).

Conclusion

Global calcium signals are derived from the co-ordinated activity of intracellular calcium channels. High resolution imaging techniques have begun to reveal that individual or small groups of channels open for a brief period to release a small pulse of calcium (Fig. 7). These events appear to have a hierarchical organization depending on stimulus intensity. Low levels of stimulation activate individual channels such as the blips recorded in *Xenopus* oocytes and HeLa cells or the quarks hypothesized to occur in cardiac cells. The next level of organization is the elementary events (puffs and sparks), which appear to be derived from small clusters of channels. At high stimulus intensities these elementary events are co-ordinated to give global events. The calcium sparks of skeletal and cardiac cells are synchronized by the action potential sweeping over the plasma membrane. In non-excitable cells, the fundamental and elementary events are co-ordinated by a process of CICR (Fig. 7) whereby the channels recruit each other to create regenerative calcium waves.

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