Another dimension to calcium signaling: a look at extracellular calcium

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Summary

Cell biologists know the calcium ion best as a vital intracellular second messenger that governs countless cellular functions. However, the recent identification of cell-surface detectors for extracellular Ca^{2+} has prompted consideration of whether Ca^{2+} also functions as a signaling molecule in the extracellular milieu. The cast of Ca^{2+} sensors includes the well-characterized extracellular-Ca^{2+}-sensing receptor, a G-protein-coupled receptor originally isolated from the parathyroid gland. In addition, other receptors, channels and membrane proteins, such as gap junction hemichannels, metabotropic glutamate receptors, HERG K^+ channels and the receptor Notch, are all sensitive to external [Ca^{2+}] fluctuations. A recently cloned Ca^{2+} sensor (CAS) in *Arabidopsis* extends this concept to the plant kingdom. Emerging evidence indicates that [Ca^{2+}] in the local microenvironment outside the cell undergoes alterations potentially sufficient to exert biological actions through these sensor proteins. The extracellular space might therefore constitute a much more dynamic Ca^{2+} signaling compartment than previously appreciated.

Key words: Parathyroid extracellular-Ca^{2+}-sensing receptor (CaR), Hemichannels, ASIC, Endocytosis, Notch, Plant Ca^{2+} sensor (CAS)

Introduction

Life forms, from vertebrate animals, to plants, to *Paramecium* exploit the unique physical and chemical properties of the calcium ion to carry out essential biological functions (Berridge et al., 2000). Several characteristics (including a favorable ionic radius and hydration status, an irregular geometry, and flexible coordination chemistry) allow it to interact and reversibly bind to specific intracellular protein targets (Yang et al., 2002). Thus, Ca^{2+} has assumed indispensable roles in intracellular signaling and as a structural component of living organisms. Many organisms also express cell-surface sensors for Ca^{2+}. Some of these proteins might have originally evolved as part of a general class of nutrient and mineral ion sensors that, with time, acquired new functions in their multicellular hosts. In particular, it appears that cells might have found a way to use these proteins to extend the signaling properties of the highly versatile Ca^{2+} to an additional dimension: outside the cell.

Interest in extracellular Ca^{2+} has been (and probably always will be) eclipsed by the immense attention granted to the second messenger function of Ca^{2+} inside the cell (Berridge et al., 2003; Berridge et al., 2000). This stemmed from the development of reliable indicators for intracellular Ca^{2+}, starting in the 1960s with the use of the Ca^{2+}-sensitive luminescent photoprotein aequorin. Subsequent advances included the introduction of fluorescent small molecule indicators that can be loaded non-invasively into cells, such as fura-2-AM and fluo-3-AM (Rudolf et al., 2003), and green fluorescent protein (GFP)-based indicators targeted to discrete cellular domains that allow Ca^{2+} measurements in specialized subcellular compartments and microdomains (Nagai et al., 2001; Zhang et al., 2002).

In general, fluorescence measurements (and patch clamp measurements) are much more conveniently performed on isolated cells in a dish, rather than on thick, optically inaccessible tissues. Perhaps it is for this reason that another potentially interesting Ca^{2+} signaling compartment, namely the local microenvironment immediately adjacent to the exterior of the cell, has been largely overlooked. In multicellular animals, the volume of interstitial fluid surrounding most cells is exceptionally small. However, gradients of Ca^{2+} that might be generated transiently in the external microenvironment of an intact tissue should rapidly dissipate in the isolated cell models typically used for fluorescence and electrophysiological measurements.

The identification of specific detectors on cells for extracellular Ca^{2+} has prompted investigation of whether external [Ca^{2+}] undergoes dynamic fluctuations under physiological conditions in intact tissues. Here, I discuss these sensors, consider the mounting evidence that local extracellular Ca^{2+} levels do indeed fluctuate, particularly in association with intracellular Ca^{2+} signaling events and neuronal activity, and address the possibility that these fluctuations constitute a unique form of Ca^{2+} signaling.

When does [Ca^{2+}] change in the external microenvironment?

Cells as Ca^{2+} sources and sinks during intracellular Ca^{2+} signaling events

In humans and many other animals, the parathyroid gland, bones, renal system and intestine all cooperate to balance the uptake, excretion and recycling of Ca^{2+} in our bodies. Thanks to the extraordinary surveillance by these homeostatic

mechanisms, Ca^{2+} in the blood is maintained at very consistent levels (~1.4 mM) in a normal individual (Brown and MacLeod, 2001). Despite the constant concentration of serum Ca^{2+}, one can observe local fluctuations in the restricted volume of interstitial fluids bathing cells (Caroppo et al., 2001; Keicher et al., 1991; Perez-Armendariz and Atwater, 1986; Pumain and Heinemann, 1985; Rusakov and Fine, 2003). One instance when this is very likely to occur is during intracellular Ca^{2+} signaling events. Indeed, prior to the development of fluorescent indicators, many of the classical physiological experiments on hormone-evoked Ca^{2+} signals used measurements of $^{45}Ca^{2+}$ fluxes in intact tissues and organs. Some of the first clues that hormones and neurotransmitters could evoke intracellular Ca^{2+} signals were provided by observations of the significant extracellular depletion or accumulation of the cation upon stimulation of whole tissues by agonists (Nielsen and Petersen, 1972).

Cells become major sources and sinks for Ca^{2+} during cytoplasmic Ca^{2+} signaling events owing to the activation of Ca^{2+} export (e.g. by the plasma membrane Ca^{2+}-ATPase, PMCA) and Ca^{2+} entry (e.g. by store-operated channels, SOCs) across the plasma membrane. Although these two processes ultimately balance as cellular $[Ca^{2+}]$ is restored back to resting levels upon signal termination, transient gradients of extracellular $[Ca^{2+}]$ can be generated wherever there is temporal and/or spatial segregation of Ca^{2+} influx and efflux (Ashby and Tepikin, 2002; Belan et al., 1997; Peng et al., 2003; Petersen, 2003). For example, Caroppo, Gerbino and colleagues used Ca^{2+}-selective microelectrodes advanced into the interstitial spaces between gastric epithelial cells and in the lumen of the gastric gland in an intact stomach to measure changes directly in extracellular $[Ca^{2+}]$ secondary to intracellular Ca^{2+} spikes stimulated by cholinergic agonists (Caroppo et al., 2004). Owing to the polarized distribution of Ca^{2+} entry and export pathways in these cells, transient increases in $[Ca^{2+}]$ of up to 0.5 mM were recorded in the limited luminal spaces of the gastric gland, whereas similar reductions in $[Ca^{2+}]$ were monitored at the basolateral aspect of the cells (Fig. 1). Large (~0.5 mM) reductions in $[Ca^{2+}]$ in the intercellular spaces of pancreatic islets were also reported by Perez-Armendariz and Atwater, who measured extracellular $[Ca^{2+}]$ with microelectrodes following stimulation of islet cells with glucose (Perez-Armendariz and Atwater, 1986).

Extracellular $[Ca^{2+}]$ depletion in excitable cells

Excitable cells have, in addition to the above-mentioned Ca^{2+} influx and efflux mechanisms, a variety of voltage-dependent Ca^{2+} entry pathways that probably influence extracellular $[Ca^{2+}]$ during neuronal activity (Cohen and Fields, 2004; Egelman and Montague, 1999; Vassilev et al., 1997). In the central nervous system, synchronous opening of voltage-operated Ca^{2+} channels (VOCCs) has been demonstrated to stimulate measurable, sometimes dramatic, reductions in extracellular $[Ca^{2+}]$ (Pumain and Heinemann, 1985; Rusakov and Fine, 2003). Using Ca^{2+}-selective microelectrodes, Pumain and Heinemann recorded $[Ca^{2+}]$ reductions from a basal level of 1.25 mM to as low as 0.08 mM in rat neocortex following application of excitatory amino acids (glutamate or aspartate) (Pumain and Heinemann, 1985). In cardiac muscle, a metallochromic absorbance-based Ca^{2+} indicator has revealed transient depletions in external $[Ca^{2+}]$ of ~200 μM during a single heartbeat, averaged over the entire heart (Cleemann et al., 1984).

Local $[Ca^{2+}]$ might fluctuate to an even greater extent in specific microdomains (e.g. t-tubules) than these tissue-averaged measurements indicate, or in other restricted

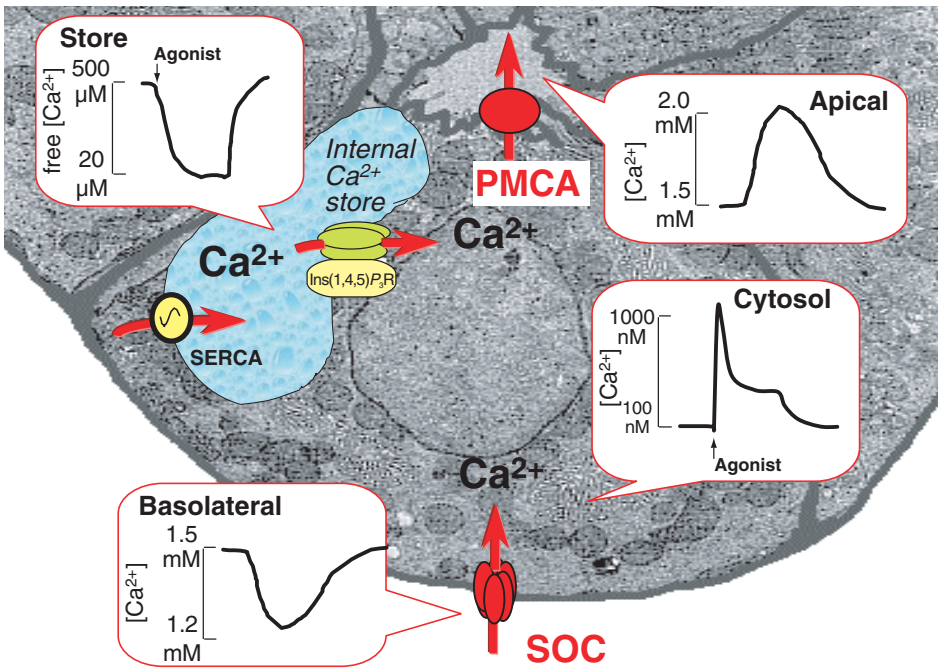

Fig. 1. Relationship between intracellular and extracellular $[Ca^{2+}]$ changes in polarized gastric epithelial cells. Intracellular inositol (1,4,5)-trisphosphate [Ins(1,4,5)P_3]-sensitive internal stores contain large amounts of Ca^{2+}. Stimulation of cells with an Ins(1,4,5)P_3-generating agonist results in the release of Ca^{2+} into the cytoplasm, and a drop in free intraluminal $[Ca^{2+}]$ in stores of several hundred μM. The resulting Ca^{2+} spike in the highly buffered cytoplasmic milieu elevates free intracellular $[Ca^{2+}]$ from a resting value of ~100 nM to ~1000 nM. Much of this Ca^{2+} is rapidly extruded by Ca^{2+}-export mechanisms, such as the plasma membrane Ca^{2+}-ATPase (PMCA) pump, which has an apical localization in these cells. Extracellular $[Ca^{2+}]$ in the restricted luminal domain of gastric glands transiently increases by hundreds of μM as a consequence of Ca^{2+} extrusion. Concomitantly, store depletion triggers the opening of store-operated channels (SOCs) in the basolateral plasma membrane and the resulting Ca^{2+} influx depletes extracellular Ca^{2+} in the limited interstitial spaces by several hundred μM (Caroppo et al., 2001; Hofer et al., 2004). When Ins(1,4,5)P_3 production is terminated, reuptake of Ca^{2+} into internal stores mediated by the sarco-endoplasmic reticulum ATPase (SERCA) causes Ca^{2+} entry to cease.

diffusion-limited spaces inaccessible to microelectrodes, such as the synaptic cleft. Fluorescence imaging of a low-affinity Ca^{2+} indicator (Fluo-5N) trapped within sealed t-tubules of skeletal muscle showed that luminal [Ca^{2+}] drops from 1.5 mM to 48 μM in 30 seconds following stimulation of Ca^{2+} entry into the cell (Launikonis et al., 2003). Although Ca^{2+} exchange between the t-tubule and the bulk solution will mitigate this drop in vivo, these results nevertheless highlight the extremely limited nature of this extracellular Ca^{2+} pool in intact muscle.

Ca^{2+} sensors in animal cells

The extracellular-Ca^{2+}-sensing receptor (CaR)

What are the Ca^{2+}-sensing mechanisms in animal cells? By far the best understood Ca^{2+} sensor is the extracellular-Ca^{2+}-sensing receptor (CaR), a G-protein-coupled receptor that responds to ambient di- and poly-valent cations. The properties of this unique receptor, which was originally cloned from the bovine parathyroid gland (Brown et al., 1993), have been reviewed extensively elsewhere (Hofer and Brown, 2003; Yano et al., 2004) (reviewed by Brown and MacLeod, 2001). It is expressed in varying amounts in many diverse tissues throughout the body. In mammals, high levels of expression have been reported in epithelial cells (e.g. of the gut and kidney), central and peripheral neurons (including enteric neurons), and also in glial cells in the brain (Brown and MacLeod, 2001; Hebert et al., 2004; Yano et al., 2004). CaR is concentrated in nerve terminals (Ruat et al., 1995) and is often restricted to particular domains of polarized cells (e.g. apical versus basolateral) (Hebert et al., 2004; Hofer et al., 2004; Sands et al., 1997). The same receptor has been identified in a wide variety of organisms, ranging from mammals, amphibians, birds and fish, and there is functional evidence for a CaR-like receptor in *Paramecium* (Klauke et al., 2000).

Human inherited disorders caused by activating and inactivating CaR mutations and mouse knockout models (Ho et al., 1995; Hough et al., 2004; Pollak et al., 1993; Pollak et al., 1994) illustrate the vital role of the receptor in maintaining normal systemic Ca^{2+} homeostasis. Activation of CaR inhibits secretion of parathyroid hormone from the parathyroid gland; principally for this reason, complete ablation of functional CaR is lethal, resulting in severe skeletal demineralization, extremely high serum [Ca^{2+}], growth defects (Ho et al., 1995) and ultra-structural changes in the epidermis (Komuves et al., 2002). CaR-knockout mice have been generated in which normal parathyroid hormone secretion has been rescued (Kos et al., 2003; Tu et al., 2003). These animals survive, and manifest more-subtle phenotypic disturbances, such as defects in renal Ca^{2+} handling. A mutant mouse strain containing an activating mutation in CaR has been reported to be associated with the formation of cataracts, inappropriate tissue calcification, and high rates of sudden death (Hough et al., 2004).

CaR is activated not only by Ca^{2+} but also by other divalent and trivalent metal ions (e.g. Mg^{2+}, Zn^{2+} and Gd^{3+}) and a wide range of biologically relevant polycations such as spermine, spermidine, neomycin and other aminoglycoside antibiotics (Breitwieser et al., 2004). Significantly, CaR is also a physiological sensor of extracellular amino acids, particularly those that have aromatic side-chains (Conigrave et al., 2000a; Conigrave et al., 2000b; Mun et al., 2004). Synthetic allosteric activators of CaR (termed calcimimetics) such as NPS-R-467 have been generated, and recent progress has also been made in the development of potent small molecule inhibitors of CaR. The latter have been termed calcilytics, because of their effects on bone remodeling mediated through an increase in parathyroid hormone secretion. Some of these drugs are currently in clinical trials for the treatment of osteoporosis (Nemeth, 2004).

Once activated by ligands, CaR couples to a complex array of intracellular signal transduction cascades (Hofer and Brown, 2003; Ward, 2004). CaR-transfected HEK 293 cells are the most commonly used model, and these cells seem to reproduce faithfully many of the signaling characteristics of the parathyroid cell, on which CaR is extremely abundant. However, the signaling mechanisms can vary depending on the cellular context. Some features of CaR-mediated signal transduction pathways in HEK CaR cells and parathyroid cells are shown in Fig. 2.

Ca^{2+} as an autocrine or paracrine messenger working through CaR

Since extracellular Ca^{2+} levels fluctuate adjacent to the cell surface as a consequence of intracellular Ca^{2+} signaling, can CaR, located on the same or on a neighboring cell, respond to these changes? In co-cultures of HEK CaR-bearing 'sensor' cells and other 'donor' cells (e.g. fibroblasts) grown under an artificial polypropylene mesh to minimize the volume of extracellular fluid, stimulation of donor cells with a Ca^{2+}-mobilizing agonist that does not bind to the sensor cells activates adjacent HEK CaR-bearing sensor cells (Hofer et al., 2000; Thomas, 2000). Additional experiments have revealed that CaR on the sensor cells responds to Ca^{2+} exported from the donor cells during Ca^{2+} signaling. Extracellular Ca^{2+} might thus act as a paracrine messenger via CaR to communicate information about the Ca^{2+} signaling status of neighboring cells.

Further work has provided evidence for an autocrine mode of extracellular Ca^{2+} signaling during CaR-mediated intracellular [Ca^{2+}] oscillations. Pulsed export of Ca^{2+} during intracellular Ca^{2+} spiking has been described previously (Belan et al., 1998; De Luisi and Hofer, 2003), and this might repetitively stimulate CaR on the same or a neighboring cell (Gracheva and Gunton, 2003). Since intracellular [Ca^{2+}] oscillates vigorously in HEK CaR cells during CaR activation (Breitwieser and Gama, 2001; De Luisi and Hofer, 2003; Young and Rozengurt, 2002), export of Ca^{2+} following a cytoplasmic Ca^{2+} spike might promote the subsequent spike by stimulating CaR on the cell surface. Experiments using extracellular Ca^{2+} buffers, inhibitors of Ca^{2+} export, and direct measurements of the time course of extracellular Ca^{2+} oscillations indicated that Ca^{2+} cycling indeed reinforces intracellular Ca^{2+} spiking (De Luisi and Hofer, 2003), thereby making CaR more 'excitable' (Fig. 3).

Does this mode of paracrine/autocrine signaling have any functional significance? Caroppo et al. have examined the ramifications of the asymmetrical physiological changes in extracellular [Ca^{2+}] induced by cholinergic stimulation (described in Fig. 1) on two secretory functions of the amphibian gastric mucosa (Caroppo et al., 2004). The glandular epithelial cells of the stomach express CaR on their

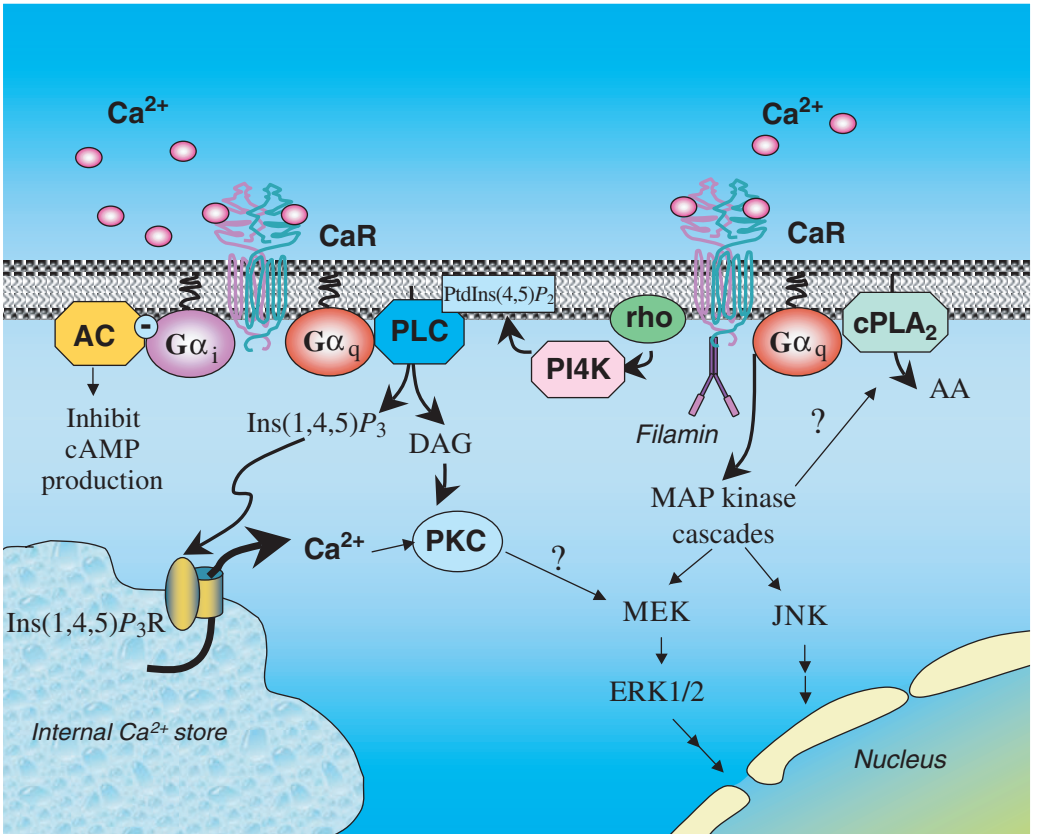

Fig. 2. Signal transduction and CaR. CaR intersects with two classical signaling pathways, working through $G\alpha_q/G\alpha_{11}$ to stimulate intracellular PLC/Ins(1,4,5)P_3/Ca^{2+} signaling, and through PTX-sensitive $G\alpha_i$ to interrupt cAMP production. In addition, CaR is linked to AA production, and various MAP kinase pathways. Abbreviations: AA, arachidonic acid; AC, adenylate cyclase; cAMP, cyclic AMP; DAG, diacylglycerol; ERK, extracellular signal-regulated protein kinase; Ins(1,4,5)P_3, inositol (1,4,5)-trisphosphate; Ins(1,4,5)P_3R, inositol (1,4,5)-trisphosphate receptor; JNK, Jun NH_2-terminal kinase; MAP kinase, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; $cPLA_2$, cytosolic phospholipase A_2; PI4K, phosphoinositide 4-kinase; PKC, protein kinase C; PLC, phospholipase C; PtdIns(4,5)P_2, phoshatidylinositol (4,5)-bisphosphate; PTX, pertussis toxin.

apical surface (Caroppo et al., 2001); the receptor therefore appears poised to detect the extracellular [Ca^{2+}] increase that occurs in the luminal compartment secondary to intracellular Ca^{2+} signaling events. Alkaline and pepsinogen secretion are well-established consequences of cholinergic stimulation of the gastric epithelium. These activities have been assumed to be the result of the intracellular Ca^{2+} signal elicited through cholinergic stimulation. However, Caroppo and colleagues found that simultaneous elevation of luminal [Ca^{2+}] and reduction of basolateral [Ca^{2+}] (previously shown to occur following carbachol stimulation) could fully reproduce these two secretory functions in the intact gastric mucosa. Remarkably, both the apical [Ca^{2+}] increase and basolateral decrease are required to achieve the full effect. Additional experiments showed that the modest extracellular [Ca^{2+}] changes following carbachol stimulation are both necessary and sufficient to elicit secretion, independently of intracellular Ca^{2+} signaling. Stimulation of apical CaR appears to result in inhibition of cAMP signaling in the epithelial cells, but we do not know how the basolateral decrease is detected and translated into the functional response. The existence of another type of Ca^{2+} sensor on the basolateral side of the cell is an intriguing possibility. These results suggest that cells can take advantage of the obligate cycling of Ca^{2+} between intracellular and extracellular compartments during agonist stimulation, and use Ca^{2+} as an extracellular 'third' messenger to direct different subsets of tissue functions.

Other Ca^{2+} sensors and proteins sensitive to external Ca^{2+}

Although CaR is undeniably the best-studied extracellular Ca^{2+} detector, there are several other membrane proteins that alter their function in response to physiologically relevant shifts in external [Ca^{2+}]. The following is a partial list of proteins that might potentially fulfill specific roles as extracellular Ca^{2+} sensors in vivo, but this has not been formally established in most of these CaRs.

Metabotropic glutamate receptors

Metabotropic glutamate receptors (mGluRs) are G-protein-coupled receptors expressed mostly in the central nervous system that are structurally similar to CaR (Hermans and Challiss, 2001). Certain mGluR isoforms (mGluR1 and mGluR5) have been reported to possess significant sensitivity to extracellular Ca^{2+} (Francesconi and Duvoisin, 2004; Kubo et al., 1998). It is not entirely clear whether these mGluRs exist

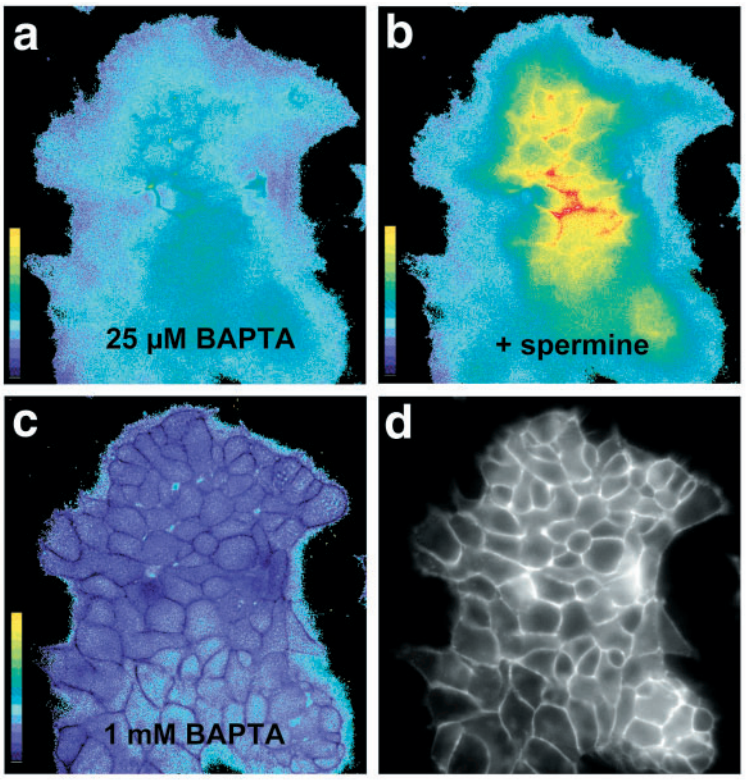

Fig. 3. Extracellular [Ca^{2+}] measured with a near-membrane fluorescent indicator in CaR-expressing HEK 293 cell clusters. The figure shows results of experiments in which cells were initially bathed in a low [Ca^{2+}] solution containing a low concentration of the high-affinity Ca^{2+} chelator BAPTA (25 μM; a). This allowed detection of extracellular [Ca^{2+}] changes by a high-affinity near-membrane fluorescent Ca^{2+} indicator, fura-C_{18}. Stimulation of intracellular Ca^{2+} signaling through CaR using 1 mM spermine (b) results in an increase in the fura-C_{18} ratio (red pseudocolor) in the diffusion-limited clefts between cells, which is indicative of an increase in extracellular [Ca^{2+}]. Extracellular [Ca^{2+}] is reduced even further in the presence of 1 mM BAPTA (c). (d) Membrane localization of the fluorophore (fluorescence at 340 nm excitation, emission 510 nm). For additional details, see De Luisi and Hofer (De Luisi and Hofer, 2003).

in nature as pure Ca^{2+} sensors (Francesconi and Duvoisin, 2004), or whether extracellular Ca^{2+} just sensitizes the receptor to its principal ligand, the excitatory amino acid L-glutamate (Nash et al., 2001; Tabata and Kano, 2004). Nevertheless, it is notable that CaR can detect L-amino acids and, conversely, mGluR can in effect sense extracellular [Ca^{2+}] changes. The molecular binding pocket that permits amino acid sensing might be conserved in both receptors (Mun et al., 2004). Another structurally related receptor for amino acids, the $GABA_B$ receptor, is likewise susceptible to changes in extracellular [Ca^{2+}] (Wise et al., 1999). For both mGluR and $GABA_B$ receptors, it remains to be seen whether alterations in external [Ca^{2+}] within the central nervous system have physiological or pathological effects on receptor activity.

Hemichannels

Gap junctions are formed by the docking of two hemichannels, one contributed from each of two adjacent cells. Functional hemichannels can exist in isolation in the plasma membrane and can mediate rapid exchange of small molecules between the inside and outside of the cell. Quist et al. and others have shown that opening of these channels is highly responsive to small decreases in extracellular [Ca^{2+}] (~200 μM) around the physiological Ca^{2+} set point of 1.8 mM (Ebihara et al., 2003; Quist et al., 2000). A Ca^{2+}-binding site in the vestibule of the connexin 32 hemichannel accounts for the regulatory effect of external Ca^{2+}. This particular residue is mutated in one form of a hereditary peripheral neuropathy, X-linked Charcot-Marie-Tooth disease (Gomez-Hernandez et al., 2003). High-resolution surface mapping of reconstituted connexin 43 hemichannels using atomic force microscopy shows that the pore exists in either an open (~2.5 nm diameter) or closed (~1.8 nm diameter) conformation, and the open probability of the pore is strongly dependent on external [Ca^{2+}] over a range of 1.8-1.0 mM (Thimm et al., 2005). In an intriguing twist to the hemichannel story, important cellular constituents such as ATP and glutamate can be liberated into the extracellular milieu through open hemichannels, where they can exert paracrine actions on purinergic or glutamatergic receptors (Parpura et al., 2004; Stout et al., 2002). Thus, one can envisage an interesting scenario in which a local reduction in [Ca^{2+}] (e.g. following synchronous neuronal activity) results in activation of diverse signaling cascades through the local release of these paracrine messengers.

Ion channels

A growing number of ion channels, particularly those native to neuronal and glial cell types, are now known to respond to fluctuations in extracellular [Ca^{2+}]. Among these are the ASIC1a/ASIC1b, proton-gated cation channels permeable to Na^+ and Ca^{2+} found in central and peripheral nerves (Babini et al., 2002). Interestingly, these are modulated by modest changes not only in extracellular Ca^{2+} but also in extracellular Mg^{2+} and polycations such as spermine. Xiong et al. recently provided evidence that ASIC1a is the key link between ischemia, acidosis and the injurious effects of ischemia in the brain, owing to the influx of toxic levels of Ca^{2+} into the cells during activation of the proton-gated channel (Xiong et al., 2004). Note that ischemia might be a condition under which derangements in extracellular [Ca^{2+}], as well as interstitial pH, also occur (Imadojemu et al., 2004).

Novel non-selective cation channels sensitive to external Ca^{2+} have also been identified in hippocampal neurons (Xiong

et al., 1997). These 36-pS channels are mostly silent in 2.0 mM extracellular [Ca^{2+}] but become strongly activated by modest stepwise [Ca^{2+}] decreases, the current at about 50% of maximum in 0.15 mM Ca^{2+}. These channels are susceptible to the anti-epileptic drug lamotrigine, which is significant because the synchronous neuronal activity associated with seizures is a condition under which rather dramatic decreases in interstitial [Ca^{2+}] levels are known to occur (Xiong et al., 2001). Another class of ion channels unusually sensitive to modest fluctuations in external [Ca^{2+}] are the HERG channels, voltage-gated K^+ channels implicated in cardiac arrhythmia (Johnson et al., 2001; Sanguinetti et al., 1995). It is not known whether these channels respond in vivo to the fluctuations in extracellular [Ca^{2+}] that occur with each heartbeat. Since HERG channels are in effect blocked by high [Ca^{2+}], it is tempting to speculate that the local Ca^{2+} depletion outside the myocyte during contraction allows the K^+ channel to be more active, thereby facilitating the repolarization phase of the cardiac contractile cycle.

As mentioned above, the synaptic cleft is a site in which considerable reductions in extracellular [Ca^{2+}] probably occur (Cohen and Fields, 2004). Study of processes potentially regulated by intra-cleft Ca^{2+} has been hampered by the inaccessibility of the membrane surfaces inside the cleft. Smith et al. have now directly patch clamped synaptosomes, structures derived from pinched-off cortical nerve terminals, identifying a novel nonselective cation channel that responds to physiological decreases in external [Ca^{2+}] (Smith et al., 2004). Several pharmacological properties of this prominent conductance are suggestive of indirect regulation through a CaR-like receptor. Smith et al. suggest that the sensitivity of this presynaptic channel to drops in external [Ca^{2+}] could represent a novel form of retrograde signaling. Ca^{2+} entry into the postsynaptic terminal during neurotransmission could reduce [Ca^{2+}] in the synaptic cleft, which would then be detected presynaptically through this mechanism.

Channel activity might not be the only neuronal process regulated by fluctuations in extracellular [Ca^{2+}] in the synaptic cleft. Using a temperature trick to separate temporally endo- and exocytosis in garter snake motor neuron boutons, Teng and Wilkinson (Teng and Wilkinson, 2003) found increased rates of endocytosis when extracellular [Ca^{2+}] was elevated incrementally over the range of 0-7.2 mM. These effects were independent of intracellular [Ca^{2+}]. Complete removal of extracellular Ca^{2+} does not entirely abolish endocytosis, which suggests a regulatory role for external Ca^{2+} in vesicle recycling rather than a strict requirement for Ca^{2+}.

Notch as a sensor of extracellular Ca^{2+} in asymmetric patterning in vertebrates

Vertebrates are asymmetrical around their left-right body axis, but how this bias is generated during embryogenesis is not completely understood. Raya et al. have provided evidence for the involvement of a persistent extracellular [Ca^{2+}] gradient in establishing asymmetric patterns of gene expression in chick embryos (Raya et al., 2004). This gradient in [Ca^{2+}] targets a cell-surface receptor, Notch, the activity of which is enhanced in the presence of elevated [Ca^{2+}]; this results in asymmetric secretion of the signaling molecule Nodal on the left side of the developing embryo. Disruption of the extracellular Ca^{2+} gradient also perturbs establishment of left-right patterning as the embryos mature.

Ca^{2+} sensors in plants

As is the case in animal cells, intracellular Ca^{2+} is also an important second messenger in the plant kingdom. Complex signals such as Ca^{2+} oscillations are used by plant cells to direct particular cellular behaviors – for example, intracellular spiking in guard cells elicited by various types of stimuli can trigger closure of adjacent stomata, pores in the epidermis through which gas and water exchange occur (Allen et al., 2001; McAinsh et al., 1995). Physiological levels of extracellular Ca^{2+} in plants are in the sub-millimolar range but have been predicted to fluctuate depending on the rate of transpiration. McAinsh and colleagues showed that a shift in apoplastic (extracellular) [Ca^{2+}] from 10 μM to 100 μM leads to regular intracellular Ca^{2+} oscillations correlated with stomatal closure in *Commelina* (McAinsh et al., 1995). Acutely reducing apoplastic [Ca^{2+}] to very low levels also results in the generation of periodic Ca^{2+} spiking inside aequorin-expressing tobacco plant cells (Cessna and Low, 2001). These results suggest a Ca^{2+} detector exists on the plant cell surface and communicates information about the extracellular [Ca^{2+}] to the cell interior.

Very recently, definitive progress has been made towards the molecular identification of an extracellular Ca^{2+} receptor in plants. Han et al. showed that increased external [Ca^{2+}] also causes intracellular Ca^{2+} signaling in *Arabidopsis* guard cells (Han et al., 2003), as had been shown previously in *Commelina* (McAinsh et al., 1995). They went on to use an expression cloning strategy employing a human cell line (HEK 293) transfected with *Arabidopsis* genes to screen for intracellular Ca^{2+} responses following a challenge with increased extracellular [Ca^{2+}]. This allowed the group to identify a 387-residue *Arabidopsis* plasma membrane protein, CAS (Ca^{2+}-sensing receptor). No obvious ortholog exists in any animal species; yet CAS can couple to the phosphoinositide/Ca^{2+}-signaling pathway in the mammalian host cell (CAS apparently increases intracellular [Ca^{2+}] in plants by using this same pathway). CAS appears to traverse the membrane only once, in contrast to the seven-transmembrane-span regions typical of CaR and other G-protein-coupled receptors. CAS, which is expressed in leaves, stems, flowers and guard cells, has a central role not only in stomatal closure, but also in the bolting response (the process whereby a plant sends up a flowering shoot). As was the case with CaR, the molecular identification of CAS should facilitate future investigation of the physiological roles of extracellular Ca^{2+} in plants.

Conclusions and perspectives

Biological signaling systems consist of at least three basic elements: a signal, a sensor and a means to link the activated sensor with a biological output. The occurrence of dynamic alterations in extracellular [Ca^{2+}] in intact tissues (the signal) and the expression of cell-surface proteins poised to detect these fluctuations (the sensors) are highly suggestive of an alternative form of Ca^{2+} signaling that takes advantage of extracellular, rather than intracellular, [Ca^{2+}] changes. The sensors range from the very well characterized CaR in animal cells to the recently described CAS in plants. Ca^{2+} efflux and

influx associated with neuronal activity or hormone stimulation are two conditions in which extracellular [Ca^{2+}] probably undergoes dynamic fluctuations. However, we still know relatively little about how these changes in interstitial [Ca^{2+}] are ultimately translated by Ca^{2+} sensors into biological outputs, or even what those outputs are. This should prove to be an exciting area of inquiry in the future.

Numerous cellular processes and extracellular proteins in addition to those described in this review can be affected by alterations in extracellular [Ca^{2+}]. Unfortunately, in many studies, drastic shifts in [Ca^{2+}] (from highly buffered EGTA-containing Ca^{2+}-free solutions to those containing tens or hundreds of mM Ca^{2+}) have been used to test the Ca^{2+} sensitivity of the process under investigation. [Ca^{2+}] changes of this extent are unlikely to occur in nature. As information about the magnitude of extracellular [Ca^{2+}] fluctuations in intact biological systems accrues, it will be interesting to revisit some of these Ca^{2+}-sensitive processes to see whether they respond to physiological alterations in extracellular [Ca^{2+}].

Of course our current grasp of the dynamic characteristics of the extracellular Ca^{2+} 'signal' is rudimentary, at best. Comprehensive understanding of where, how and when Ca^{2+} fluctuates will require greatly improved methods for quantitatively monitoring [Ca^{2+}] in the extracellular microenvironment. By analogy with the tremendous developments made in the field of intracellular Ca^{2+} signal transduction following the introduction of fluorescent Ca^{2+} dyes, sensitive imaging techniques that allow high spatiotemporal resolution of extracellular [Ca^{2+}] fluctuations would be very desirable. The ideal probe would have an appropriate Ca^{2+} affinity and sensitivity to distinguish [Ca^{2+}] changes against a background of physiological [Ca^{2+}], and could be targeted to specific extracellular domains. In addition it should be detectable in vivo, in which normal tissue architecture and blood circulation are maintained. Unfortunately, fluorescent indicators are at the practical limit of usefulness in this regard; even with sophisticated multiphoton imaging techniques, visible light cannot penetrate more than 0.5 mm deep into tissues, which precludes the use of this approach for many organ systems. Ultimately, we will need to think beyond conventional fluorescence and optical techniques (Tsien, 2003) if a genuine comprehension of the first (and 'third') messenger function of extracellular Ca^{2+} is to be appreciated in the future.

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