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Extracellular calcium as an integrator of tissue function

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Abstract

The past several decades of research into calcium signaling have focused on intracellular calcium (Ca^{2+}_i), revealing both exquisite spatial and dynamic control of this potent second messenger. Our understanding of Ca^{2+}_i signaling has benefited from the evolution of cell culture methods, development of high affinity fluorescent calcium indicators (both membrane-permeant small molecules and genetically encoded proteins), and high resolution fluorescence microscopy. As our understanding of single cell calcium dynamics has increased, translational efforts have attempted to push calcium signaling studies back into tissues, organs and whole animals. Emerging results from these more complicated, diffusion-limited systems have begun to define a role for extracellular calcium (Ca^{2+}_o) as an agonist, spurred by the cloning and characterization of a G protein-coupled receptor activated by Ca^{2+}_o (the calcium sensing receptor, CaR). Here we review the current state-of-the art for measurement of Ca^{2+}_o fluctuations, and the evidence that fluctuations in Ca^{2+}_o can act as primary signals regulating cell function. Current results suggest that Ca^{2+}_o in bone and epidermis may act as a chemotactic homing signal, targeting cells to the appropriate tissue locations prior to initiation of the differentiation program. Ca^{2+}_i signaling-mediated Ca^{2+}_o fluctuations in interstitial spaces may integrate cell signaling responses in multicellular networks through activation of CaR. Appreciation of the importance of Ca^{2+}_o fluctuations in coordinating cell function will likely spur identification of additional, niche-specific Ca^{2+} sensors, and provide unique insights into the regulation of multicellular signaling networks.

Keywords

Extracellular calcium; calcium sensing receptor; multicellular network; signal integration; signal amplification

Introduction

Systemic calcium homeostasis is critical to the survival of multicellular organisms, and complex, inter-dependent regulatory systems have evolved to maintain Ca^{2+} in the extracellular fluid within a narrow range (1.1–1.4 mM Ca^{2+} for humans) (reviewed by Hurwitz, 1996). The calcium sensing receptor, CaR, is exquisitely sensitive to small changes in Ca_o^{2+} (Brown, 1983; Breitwieser & Gama, 2001). In parathyroid chief cells, this permits sensing of minute fluctuations in Ca^{2+}_o (±200 μM) (Brown, 1983), with increases in Ca^{2+}_o causing decreases in parathyroid hormone (PTH) secretion (reviewed in Ambrish & Brown, 2003). PTH has effects on the kidney to increase Ca^{2+} reabsorption from the filtrate and synthesis of vitamin D, 1,25 $(OH)_2D$ (which enhances intestinal absorption of Ca^{2+}), and on bone to increase release of

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Ca^{2+} and phosphate by demineralization. Recent reviews detail the mechanisms involved in systemic calcium homeostasis and the pathologies resulting from their dysregulation (Brown, 2004; Akerström et al., 2005; Rodriguez et al., 2005; Chattopadhyay & Brown, 2006). CaR is also expressed in many cell types which are not directly involved in systemic calcium homeostasis, including neurons and glia, endocrine and exocrine glands, epithelia, cells of hematopoietic origin, and keratinocytes (Brown and MacLeod, 2001 and references therein). Although the expression of CaR in these diverse cell types has been established at both the mRNA and protein levels, and preliminary studies have characterized Ca^{2+}_o-mediated activation of cellular signaling pathways in culture models, the physiological importance of CaR in these cell types has not be established. The current review will focus on the growing evidence that Ca^{2+}_o fluctuations occur as a routine consequence of Ca^{2+}_i signaling. Autocrine/paracrine sensing of Ca^{2+}_o fluctuations by CaR (or potentially other Ca^{2+} sensors) amplifies and integrates agonist-mediated signals in multicellular networks.

Measuring fluctuations in Ca_o^{2+}

The concentration of Ca^{2+}_o ($[Ca^{2+}_o]$) in the bulk solution bathing cells and tissues is in the range of 1.1–1.4 mM, more than 10,000 times higher than resting $[Ca^{2+}_i]$. Measurement of Ca^{2+}_o fluctuations in proximity to cells or in restricted intercellular spaces has suffered from a lack of adequate experimental tools to either access the compartment or resolve small $[Ca^{2+}]$ increments imposed on a high background $[Ca^{2+}_o]$. Ca^{2+}-sensitive small molecule indicators such as fura-2 (Haugland, 2005) and genetically encoded Ca^{2+} sensors (Palmer et al., 2006; Kotlikoff, 2007) generally respond to changes in Ca^{2+} in the nM – μM range, and are saturated at physiological $[Ca^{2+}_o]$. A variety of approaches have been used to measure $[Ca^{2+}_o]$ fluctuations, although each has its limitations with regard to either sensitivity or spatial resolution. Here we present a brief overview of the methods.

Microelectrodes filled with Ca^{2+}-ion exchange resins

Early studies of Ca^{2+}_o fluctuations utilized microelectrodes containing ion exchange resins with affinities for Ca^{2+} in the μM to mM range. Impalement of tissue with both a resin-containing microelectrode and a reference microelectrode allows measurement of changes in Ca^{2+}_o under physiological conditions, and indeed, such studies demonstrated large decreases in Ca^{2+}_o during stimulation of the central nervous system (Nicholson et al., 1977). Access to restricted diffusion compartments is possible in some isolated exocrine glands, and Ca^{2+}-selective electrodes have been used to impale gastric glands from frog mucosa (Caroppo et al., 2001; Caroppo et al., 2004) or intact gastric mucosa (Hofer et al., 2004), demonstrating slow changes in lumenal Ca^{2+}_o in response to agonist stimulation. The vibrating probe technique, which was initially developed and used to measure net charge currents in the vicinity of cells and multicellular systems (first described by Jaffe and Nuccitelli, 1974), was adapted to measuring Ca^{2+}_o gradients by using low resistance Ca^{2+}-sensitive resin-filled electrodes, vibrated across the surface of cells or tissues (Smith et al., 1994; Kűhtreiber & Jaffe, 1990). Self-referencing, vibrating Ca^{2+} microelectrodes have the sensitivity to quantify both influx and efflux in the vicinity of isolated cells, and have been used to map the Ca^{2+} fluxes near the surface of stereocilia of isolated hair cells (Yamoah et al., 1998). While the vibrating Ca^{2+}-selective probe improves spatial and temporal resolutions to a few microns and seconds, respectively, access to restricted diffusion compartments between cells is limited.

Ca^{2+}-sensitive fluorophores or genetically encoded Ca^{2+} sensors

Ca^{2+}-sensitive fluorophores have broad utility in studying the temporal and spatial dynamics of Ca^{2+} changes, but their utility for measuring Ca^{2+}_o changes has been limited to date due to their high affinities for Ca^{2+}, i.e., at normal physiological $[Ca^{2+}_o]$ most dyes are saturated (Knot et al., 2005; Knöpfel et al., 2006). Nevertheless, Ca^{2+} fluorophores afford sensitivity,

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time resolution and access to restricted spaces, prompting adaptation of fluorescence indicators to the measurement of $Ca^{2+}{}_{o}$ fluctuations. In general, such methods require experiments to be performed in limited volumes and at low [$Ca^{2+}{}_{o}$]. Impermeant Ca^{2+} dyes were used to estimate $Ca^{2+}{}_{o}$ decreases during a cardiac action potentials (Cleemann et al., 1984; Hilgemann & Langer, 1984; Hilgemann, 1986), allowing inferences about the magnitude and timing of Ca^{2+} influx into cardiac myocytes in multicellular preparations. A further iteration in the development of a reliable means of measuring $Ca^{2+}{}_{o}$ fluctuations with Ca^{2+}-sensitive fluorophores is the droplet technique, developed by Drs. Tepikin and Petersen (Tepikin et al., 1992a,b; Tepikin et al., 1994). This method makes use of current Ca^{2+}-sensitive fluorophores under low [$Ca^{2+}{}_{o}$] conditions to characterize changes in $Ca^{2+}{}_{o}$ in small clusters of exocrine gland cells. $Ca^{2+}{}_{o}$ can be measured using a Ca^{2+} indicator coupled to high molecular weight dextran beads to limit diffusion of the $Ca^{2+}{}_{o}$ signal, and $Ca^{2+}{}_{i}$ can be measured using a cleaveable, acetoxymethyl ester (AM)-conjugated dye having distinct Ca^{2+} affinities and spectral characteristics (Belan et al., 1996; Belan et al., 1998; Mogami et al., 1999). More recently, $Ca^{2+}{}_{o}$ dyes have been targeted directly to the extracellular surface of the plasma membrane using fura-C18 (De Luisi & Hofer, 2003; Hofer, 2005). Stimulation of cells can be initiated by injection of agonists/drugs into the oil droplet containing the cells, and changes in both $Ca^{2+}{}_{i}$ and $Ca^{2+}{}_{o}$ can be monitored simultaneously. While this approach has provided powerful insights into the temporal dynamics of transmembrane Ca^{2+} movements, it should be reiterated that the experiments must be performed in low or nominally zero $Ca^{2+}{}_{o}$ because of the high Ca^{2+} affinity of the fluorophores. To date, genetically encoded Ca^{2+} sensors have not been amenable to measurement of $Ca^{2+}{}_{o}$ fluctuations under physiological conditions. Engineered Ca^{2+} sensors are most commonly designed to monitor intracellular Ca^{2+} fluctuations and are based on native proteins containing high affinity, EF-hand motifs for Ca^{2+} binding (Miyawaki et al., 1999; Palmer et al., 2006; Tour et al., 2007; Kotlikoff, 2007). The power of the genetically encoded Ca^{2+} sensors includes the ability to target the sensor to distinct cellular organelles or subcellular locations (Palmer et al., 2006; Tour et al., 2007). Ca^{2+} sensors capable of accurately resolving Ca^{2+} fluctuations in the endoplasmic reticulum lumen, where [Ca^{2+}] may approach that of the extracellular environment, have been plagued by interactions with ER-resident Ca^{2+} binding proteins and/or pH effects on the sensors (Hara et al., 2004; Palmer et al., 2004; Osibow et al., 2006). A recent report of a family of Ca^{2+} sensors derived from green fluorescent protein, GFP, with affinities in the range of 0.4–2 mM, suggests that sensors useful for the real-time measurement of Ca_{o}^{2+} fluctuations in high [Ca^{2+}] intracellular compartments and at the surfaces of cells are being developed (Zou et al., 2007). The ability to genetically encode such a sensor and target it to the extracellular surface of cells or transgenic animals will greatly increase our understanding of dynamic changes of [$Ca^{2+}{}_{o}$] during cell and tissue activation.

Genesis of extracellular Ca^{2+} microdomains

[$Ca^{2+}{}_{o}$] microdomains which differ significantly from systemic [$Ca^{2+}{}_{o}$] have been identified and characterized in many tissues, including cardiac myocytes (T tubules), neurons (synaptic cleft), epithelia, regions of bone resorption, and exocrine glands. [$Ca^{2+}{}_{o}$] fluctuations in tissue microdomains can result from the differential kinetics of activity-driven movements of Ca^{2+} across the membrane, the asymmetric distribution of Ca^{2+} signaling and transport proteins in polarized cells, and/or the unique nature of the extracellular space to which Ca^{2+} movements are confined. Interstitial spaces represent a variable but minor component of tissue volume, eg. extracellular space comprises 20% of total brain volume (Thorne & Nicholson, 2006), and dense packing of cells in endocrine glands significantly increases and synchronizes hormone secretion (Petrasek et al., 2002). In most, if not all cases, all of these factors contribute to generation of $Ca^{2+}{}_{o}$ microdomains. Here we discuss the evidence for each of these contributors to physiologically relevant [$Ca^{2+}{}_{o}$] fluctuations.

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Asymmetric distribution of Ca^{2+} signaling and transport proteins

Polarized expression and targeting of Ca^{2+} permeable ion channels, the plasma membrane Ca^{2+} ATPase (PMCA) and/or the Na^+/Ca^{2+} exchanger (NCX) can create distinct plasma membrane compartments specialized for Ca^{2+} influx and efflux, potentially creating areas of extracellular accumulation or depletion of Ca^{2+}, particularly coupled with restricted diffusion spaces within a tissue. The differential distribution of Na^+, Ca^{2+} and K^+ channels, and both PMCA and NCX between cardiac sarcolemmal and T tubular membranes produces the efficient release of intracellular Ca^{2+} from the sarcoplasmic reticulum and rapid recovery of Ca^{2+}_i during diastole (Brette & Orchard, 2003), which is reflected in the reduction and recovery of $[Ca^{2+}]$ in the T tubules during each contraction (Cleemann et al., 1984; Hilgemann & Langer, 1984; Hilgemann, 1986). Similarly, the global decreases in $[Ca^{2+}_o]$ observed in intact rat brain after hypoxia (Silver & Ercińska, 1990) can be attributed to the highly specialized expression and distribution of voltage- and neurotransmitter-activated Ca^{2+} permeable channels at the pre- and postsynaptic membranes (Rusakov et al., 1999; Juhaszova et al., 2000, Wiest et al., 2000; Stanley, 2000). Indeed, the synaptic cleft represents a highly dynamic compartment for stimulus frequency-dependent Ca^{2+}_o fluctuations (Vassilev et al., 1997). The pancreatic acinar cell, a well-studied model for exocrine gland cells, takes up Ca^{2+} at the basal pole and extrudes Ca^{2+} from the apical pole after agonist stimulation (reviewed in Petersen & Tepikin, 2007). An asymmetric distribution of plasma membrane Ca^{2+} channels and PMCA, as well as distinctive subcellular distributions of intracellular organelles including mitochondria and secretory vesicles, contribute to the directional secretion of Ca^{2+} into the lumen of pancreatic acini (Belan et al., 1996; Ashby & Tepikin, 2002; Li et al., 2004). Many types of polarized epithelial cells have segregated Ca^{2+} influx and efflux pathways at apical versus basolateral membranes, leading to transepithelial Ca^{2+} transport (Bourdeau & Burg, 1980; Nellans & Goldsmith, 1981; Bronner, 1989; Friedman & Gesek, 1995; VanHouten et al., 2004).

Temporal dynamics of Ca^{2+} signaling events

The temporal dynamics of $[Ca^{2+}_i]$ changes after cell activation are critical to shaping cellular responses. Complementary changes in $[Ca^{2+}_o]$ can occur when the extracellular space is functionally compartmentalized as a result of diffusion barriers, eg. in the synaptic cleft, in the exocrine gland lumen, or along the crypt-villus axis of intestinal mucosa. Voltage activated Ca^{2+} channels generate a rapid, transient Ca^{2+} influx, which can be further potentiated by Ca^{2+}-induced Ca^{2+} release from intracellular stores. Similar rapid Ca^{2+}_i transients can be generated upon G protein-coupled receptor (GPCR) activation of Ca^{2+} release from intracellular stores. Ca^{2+}_i is then brought back to baseline levels primarily by efflux pathways including PMCA and/or NCX, with a potential second wave of targeted Ca^{2+} influx for refilling of intracellular Ca^{2+} stores. Figure 1 illustrates the molecular components of GPCR signaling via G_q, with the potential for autocrine amplification of the Ca^{2+}_i signal by CaR. In T cells, coordinate regulation of PMCA and store-sensitive Ca^{2+} release-activated Ca^{2+} channels (CRAC) shapes the intracellular Ca^{2+} transient (Bautista & Lewis, 2004). The general features of intracellular Ca^{2+} signaling dynamics for exocrine gland cells have recently been reviewed by Petersen & Tepikin (2007). Interestingly, the bulk of the $[Ca^{2+}_i]$ increase after cell activation is extruded from cells via PMCA, resulting in an estimated loss of Ca^{2+}_i on the order of 0.4 mM for exocrine secretory cells (Belan et al., 1998), with an attendant increase in Ca^{2+}_o at the apical, secretory pole of the cell (Belan et al., 1998;Petersen & Tepikin, 2007). Secretory granules themselves contain high amounts of Ca^{2+} (50–100 mM total $[Ca^{2+}]$, Hutton et al., 1983; ≈50 μM free $[Ca^{2+}]$, Mitchell et al., 2001), likely bound to the low affinity Ca^{2+} chelator chromogranin A (O'Connor et al., 1983;Yoo & Albanesi, 1990;Nicaise et al., 1992), which can be released in quanta upon initiation of secretion (Gerasimenko et al., 1996;Belan et al., 1998). Distinctions can be made between PMCA-mediated Ca^{2+} extrusion, which is non-quantal (Belan et al., 1996) and granule-mediated Ca^{2+} extrusion, which is pulsatile (Belan et al., 1998). The net effect of a strong stimulus for secretion is the transient, directional efflux

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of a significant fraction of total cellular Ca^{2+}, which can generate a physiologically relevant, transient fluctuation in $[Ca^{2+}{}_o]$.

CaR as the prototypical sensor of $Ca^{2+}{}_o$ fluctuations

The cloning of CaR (Brown et al., 1993) from bovine parathyroid laid the groundwork for an increasing awareness of the signals inherent in $Ca^{2+}{}_o$ fluctuations in cells and tissues. The highly cooperative dependence of CaR activation on $[Ca^{2+}{}_o]$ (Garrett et al., 1995; Ruat et al., 1996; Gama & Breitwieser, 1998) correlates with the steep inverse relationship between serum $[Ca^{2+}]$ and PTH secretion (Brown, 1983). CaR is expressed in tissues involved in systemic Ca^{2+} homeostasis, including kidney (reviewed in Huang & Miller, 2007), intestine (reviewed in Kirchhoff & Geibel, 2006) and bone (reviewed in Dvorak & Riccardi, 2004). CaR may also be involved in central nervous system (CNS)-mediated fine-tuning of systemic Ca^{2+} and fluid balance by regulating angiotensin secretion by the subfornical organ (Rogers et al., 1997; Washburn et al., 2000), and contributes to the regulation of Ca^{2+} transport across the placenta during fetal development (reviewed in Brown & MacLeod, 2001).

CaR is a member of family 3/C of the GPCR superfamily and functions as a disulfide-linked homodimer (Ray et al., 1999). The CaR agonist binding site lies within a large extracellular domain (ECD) consisting of a Venus flytrap-like domain (VFTD) and a cysteine-rich domain (CysRD) (Figure 2). Ca^{2+} and polyvalent cations bind in the cleft of the VFTD (Silve et al., 2005; Huang et al., 2007). Amino acids allosterically modulate CaR activation and bind to an adjacent site on the VFTD (Mun et al., 2005; Acher & Bertrand, 2005). CaR activity can also be modulated by clinically important phenylalkylamine derivatives, binding at site(s) within the transmembrane helical domain (TMD) (Petrel et al., 2003; Miedlich et al., 2004; Petrel et al., 2004). The intracellular loops (ICLs) and carboxyl terminus (CT) of CaR interact with heterotrimeric G proteins (G_q, G_i, $G_{12/13}$) to initiate signaling although the residues involved have not been unequivocally identified (reviewed in Ward, 2004). CaR also interacts with the cytoskeletal scaffold Filamin A (Awata et al., 2001; Hjalm et al., 2001), and this interaction both stabilizes CaR against degradation (Zhang & Breitwieser, 2005) and facilitates MAPK signaling (Hjalm et al., 2001; Zhang & Breitwieser, 2005). Finally, CaR has been shown to interact with RAMPs 1 and 3 (receptor-activity-modifying proteins), an interaction which is required for efficient trafficking to the plasma membrane (Bouschet et al., 2005), and may facilitate differential targeting of CaR to distinct membrane compartments. CaR is expressed in myriad cells and tissues, including neurons and glia in the CNS (Yano et al., 2004), peripheral perivascular sensory nerves (Bukoski et al., 1997), exocrine cells in the pituitary (Zivadinovic et al., 2002), pancreas (Komoto et al., 2003), and gastrointestinal tract (Conigrave & Brown, 2006; Remy et al., 2007), epithelia of the lens (Chattopadhyay et al., 1998) and olfactory bulb (Hubbard et al., 2002), in mammary tissue (VanHouten et al., 2004), the prostate (Lin et al., 1998), fibroblasts (McNeil et al., 1998) and keratinocytes (reviewed in Tu et al., 2004). Many of the cell types in which CaR is expressed have no known role in systemic Ca^{2+} homeostasis. What is/are the physiological roles of CaR in these diverse tissues? Family C GPCRs as a group encompass a variety of metabolic sensors for Ca^{2+}, amino acids, and tastants (Bräuner-Osborne et al., 2007). CaR is activated not only by Ca^{2+} but by amino acids and peptides binding at allosteric sites (reviewed in Breitwieser et al., 2004). Conigrave & Brown (2006) present strong arguments for a physiological role of CaR as a “taste receptor” in the gastrointestinal tract, functioning predominantly as an L-amino acid sensor. Similarly, the expression patterns for CaR in a number of fish species suggest roles in amino acid and/or salinity sensing (Naito et al., 1998; Hubbard et al., 2002; Loretz et al., 2004). For many other cells types which express CaR, the nature of the *in vivo* activating stimulus and functional consequences are unknown, although in many cases, we have a detailed map of the signaling pathways which are activated by CaR in isolated cells in culture. An increasing understanding of the dynamics of $Ca^{2+}{}_o$ fluctuations in interstitial spaces has pointed to two potential roles for CaR in integrating tissue

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function. First, CaR may act as a gradient sensor, triggering chemotaxis of motile cells to critical niches in bone or epidermis. Second, CaR may function in an autocrine/paracrine fashion to integrate cell signaling across multicellular networks, particularly in epithelia and exocrine glands. Here we discuss the mounting evidence in support of these integrative roles for CaR in tissue function.

Ca^{2+} gradients as homing signals

Ca^{2+}_o gradients are present in a number of distinct physiological niches and can represent potent signals for cell migration, bringing together the distinct cell types required to intiate a multicellular process such as bone remodeling or wound repair. CaR activates several signaling pathways which can mediate shape changes and promote cell migration, including Rho A through interactions with filamin (Pi et al., 2002), and through CaR interactions with β-arrestin-1, the Arf nucleotide binding site opener (ARNO)—ARF6—engulfment and cell motility protein (ELMO) protein network (Bouschet et al., 2007). CaR activation leads to cell migration in osteoblasts (Godwin & Soltoff, 1998; Yamaguchi et al., 1998a), peripheral blood monocytes (Yamaguchi et al., 1998b; Olszak et al., 2000), stem cells (Adams et al., 2006), and GnRH neurons (Chattopadhyay et al., 2007). CaR also plays a role in fibroblast and keratinocyte migrations during epidermal development and wound repair (reviewed by Martin, 1997; Landsdown, 2002).

Bone remodeling occurs constantly at 1–2 million discrete sites in the adult skeleton (reviewed in Raisz, 1999). Bone remodeling at each site is initiated by osteoclasts, multinucleated cells derived monocytes (reviewed by Teitelbaum, 2000). Bone demineralization is achieved by vectorial secretion of HCl into the resorptive space formed under the mature osteoclast, yielding an extracellular pH < 4.5 and very high local [Ca^{2+}_o], on the order of 40 mM (Silver et al., 1988). Multiple cell types in bone marrow express CaR (House et al., 1997), and in particular, stromal cells (Yamaguchi et al., 1998a) and osteoblasts (Sugimoto et al., 1993); Godwin & Soltoff, 1997) are activated by high [Ca^{2+}_o] and migrate in response to Ca^{2+} gradients. While there has been some dispute in the literature regarding which cell types that contribute to bone remodeling express CaR, or a related, recently deorphanized receptor, GPRC6A (discussed in Brown, 2007), it is clear that Ca^{2+}_o represents a potent homing signal which targets cells required for bone formation to the site(s) of bone dissolution. Interestingly, both CaR and GPRC6A are activated by divalent cations and amino acids, although each has a distinct order of amino acid preference (Conigrave et al., 2000; Christiansen et al., 2007). Sites of active bone resorption also contain high levels of amino acids from protease-mediated breakdown of bone matrix proteins (Teitelbaum, 2000), so it will be of interest to determine whether the combination of high Ca^{2+}_o and amino acids differentially affects cell migration through CaR and/or GPRC6A. High [Ca^{2+}_o] not only promotes migration of osteoblasts, but initiates a program of proliferation and maturation which is required to elaborate the gene products involved in bone matrix mineralization. Targeting constitutively active CaR to mature osteoblasts dysregulates maturation and leads to loss of cancellous bone (Dvorak et al., 2007). Finally, CaR is required for targeting of haematopoietic stem cells to the endosteal niche (Adams et al., 2006), since $CaR^{-/-}$ mice have defects in both migration and homing. CaR is being considered as a potential clinical target to enhance stem cell mobilization and engraftment in bone marrow transplants (Ballen, 2007).

A Ca^{2+}_o gradient is crucial to the normal development of skin, and to the process of wound healing. The epidermal permeability barrier is established late in fetal development (reviewed in Byrne et al., 2003). Coincident with permeability barrier formation, the Ca^{2+}_o gradient, low in basal and spinous layers and highest in the granule layer, develops (Elias et al., 1998). The Ca^{2+}_o gradient has been shown to regulate lamellar body secretion independent of barrier formation (Menon et al., 1994). *CaR*$^{-/-}$ mice have abnormalities in epidermal development,

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with enhanced numbers of proliferating keratinocytes and a decrease in expression of late markers of differentiation including filaggrin, and loricin (Komuves et al., 2002). Conversely, transgenic mice overexpressing CaR in basal cells of the epidermis, driven by the human keratin 14 promoter, have precocious fetal barrier formation, early hair follicle development, hypertrophic epidermis with an increase in spinous and granular layers and overexpression of terminal differentiation markers including filaggrin, involucrin and loricin (Turksen & Troy, 2003). CaR is thus a critical sensor of the epidermal $Ca^{2+}{}_o$ gradient during epidermal development, stimulating migration as well as initiation of the program of keratinocyte differentiation.

Wound healing is a specialized case of epidermal development, and the initiating signal includes disruption of the epidermal $Ca^{2+}{}_o$ gradient and a large increase in $[Ca^{2+}{}_o]$ in the basal layers of the epidermis. High $[Ca^{2+}{}_o]$ and/or elevated concentrations of other cations including Mg^{2+}, zinc, copper and iron, act as homing signals for monocytes (Yamaguchi et al., 1998b; Olszak et al., 2000), fibroblasts (Payne et al., 1996), and keratinocytes (Magee et al., 1987). Lateral migration into the wound site of dermal fibroblasts and keratinocytes is critical to re-epithelialization (Jensen & Bolund, 1988; Zia et al., 2000), and is initiated in response to elevated $[Ca^{2+}{}_o]$. Keratinocytes are extremely sensitive to $[Ca^{2+}{}_o]$, with the so-called "calcium switch" from proliferation to differentiation occurring when $Ca^{2+}{}_o$ is increased from 0.03 mM to >0.5 mM, while fibroblasts, in contrast, require $[Ca^{2+}{}_o]$ above 1.4 mM for proliferation (Kulesz-Martin et al., 1984). Both keratinocytes (Bikle et al., 1996) and fibroblasts (McNeil et al., 1998) require CaR expression for the $Ca^{2+}{}_o$-induced changes in cell function (Tu et al., 2001). Keratinocytes express both the normal CaR form observed in most other tissues and a splice variant with a deletion of exon 5 which does not respond to changes in $[Ca^{2+}{}_o]$ (Oda et al., 1998). During $Ca^{2+}{}_o$-induced differentiation of keratinocytes, there is a decrease in expression of the full length form of CaR, with no change in the amount of splice variant protein expressed (Oda et al., 1998). The full length form of CaR is required to initiate the "calcium switch" in keratinocytes (Komuves et al., 2002). The original $CaR^{-/-}$ mouse, which utilized an insertion of the neomycin cassette into exon 5 (Ho et al., 1995) represents an interesting control, since it expresses only the splice variant. Epidermal differentiation in $CaR^{-/-}$ mice is limited, with enhanced proliferation despite an enhanced epidermal Ca^{2+} gradient (Komuves et al., 2002), confirming the importance of full length CaR for initiation of keratinocyte differentiation.

Ca^{2+} fluctuations as integrators of tissue activity

Hofer and colleagues have championed the idea that CaR acts as a sensor and integrator of tissue activity, since their first description of intercellular communication via CaR (Hofer et al, 2000). This was a revolutionary idea, eliciting a "News and Views" comment by Thomas (2000), and shifted the focus from intra- to extracellular Ca^{2+} as a signal for tissue integration. To this point, tissue integration of Ca^{2+} signals was considered predominantly a phenomenon linked to gap junctions. HEK293 cells stably expressing human CaR (HEK-CaR) were cultured on coverslips under restricted diffusion conditions (fine gauge polypropylene fibers were melted onto glass coverslips to generate small lacunae between the plastic and the glass). CaR activation by varying $[Ca^{2+}{}_o]$, or upon addition of an allosteric agonist for CaR, NPS R-467, elicited characteristic alterations in $[Ca^{2+}{}_i]$. To test for Ca^{2+}-mediated intercellular communication, HEK-CaR cells were co-cultured with non-CaR expressing cells (either BHK-21 fibroblasts or freshly isolated pancreatic acinar cells). Agonist-mediated activation of an increase in $[Ca^{2+}{}_i]$ in BHK-21 cells was elicited by histamine, which induced an alteration in $[Ca^{2+}{}_i]$ of adjacent HEK-CaR cells after a brief time lag. Similar experiments with pancreatic acinar cells elicited HEK-CaR cell changes in $[Ca^{2+}{}_i]$ in response to cholecystokinin and/or bombesin, activators of pancreatic cell receptors. Activation of HEK-CaR cells required both close proximity to BHK-21 fibroblasts or pancreatic acinar cells, and restricted diffusion, i.e.,

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location of the cells in lacunae created by the polyethylene fibers. Incubation of the agonist-activated cells with BAPTA-AM, to buffer the rise in [$Ca^{2+}{}_i$] and limit Ca^{2+} efflux, or inclusion of a Ca^{2+} buffer (citrate/isocitrate/BAPTA) in the extracellular solution inhibited the responses in the HEK-CaR cells. These results demonstrated that agonist activation of $Ca^{2+}{}_i$ signaling also generates a $Ca^{2+}{}_o$ signal which can be "read" by adjacent cells which express CaR, potentially amplifying and integrating focal signals resulting from neural or paracrine pathways impinging directly onto only a subset of cells in a tissue. Figure 3 illustrates a simplified model of trans-tissue amplification by autocrine/paracrine activation of CaR by Ca^{2+} released from activated cells.

The initial experiments supporting a role for CaR in intercellular communication were performed in an artificial cell culture system (Hofer et al., 2000). Experiments in intact polarized gastric acid-secreting epithelia have demonstrated polarized alterations in [$Ca^{2+}{}_o$] in response to agonist application (Caroppo et al., 2001). Alterations in [$Ca^{2+}{}_o$] fluctuations are blocked by inhibiting release of Ca^{2+} from endoplasmic reticulum with sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitors or by chelation of intracellular Ca^{2+} with BAPTA-AM, suggesting that the [$Ca^{2+}{}_o$] fluctuations are a consequence of agonist-activated $Ca^{2+}{}_i$ signaling. Interestingly, immunostaining of the isolated tissue suggests that both PMCA and CaR are localized to basal and lateral aspects of the surface epithelial cells (Caroppo et al., 2001), putting the "sensor" in proximity to the "signal". Additional microelectrode studies in isolated epithelia (Hofer et al., 2003; Caroppo et al., 2004) confirm and extend these findings.

The potential functional linkage between CaR and PMCA was directly explored in HEK-CaR cells (De Luisi & Hofer, 2003), and results suggest that PMCA-mediated Ca^{2+} efflux increases the activity of CaR. CaR and PMCA are preferentially localized to cell contact regions, and CaR-mediated Ca^{2+} oscillations were more likely to be observed in cell clusters rather than isolated cells. Near-membrane measurements of fluctuations in [$Ca^{2+}{}_o$] using the lipophilic indicator fura-C_{18} demonstrated that increases in [$Ca^{2+}{}_o$] lagged behind increases in [$Ca^{2+}{}_i$] in response to application of spermine, an allosteric activator of CaR, suggesting that efflux was a result of CaR activation. Addition of extracellular Ca^{2+} buffers severely blunted the increase in [$Ca^{2+}{}_o$]. Ca^{2+} activation of CaR is highly cooperative, with an apparent Hill coefficient ranging from 2–4 (Garrett et al., 1995; Ruat et al., 1996; Gama & Breitwieser, 1998). These results suggest that the apparent sensitivity of CaR to $Ca^{2+}{}_o$ may be augmented by Ca^{2+} efflux from activated cells by autocrine/paracrine feedback (illustrated schematically in Figure 1), and further, that the sensitivity of CaR to [$Ca^{2+}{}_o$] fluctuations may be cell and tissue-dependent.

Overall, the studies in HEK-CaR cells make a strong case for a critical role for CaR in integrating Ca^{2+} signaling events in multicellular networks. CaR-mediated intercellular signaling requires close proximity between cells, restricted diffusion, and intact intracellular signaling pathways leading to increases in [$Ca^{2+}{}_i$] and efflux via PMCA. Parallel studies in isolated, intact epithelia suggest that the proposed signaling-mediated fluctuations in [$Ca^{2+}{}_o$] can occur. To determine the breadth and generality of CaR-mediated integration of cell signaling requires the development of methods to measure [$Ca^{2+}{}_o$] fluctuations in intact tissues. Until then, CaR double knockout mice ($CaR^{-/-}/PTH^{-/-}$ (Kos et al., 2003) or $CaR^{-/-}/Gmc2^{-/-}$ (Tu et al., 2003)) which survive to adulthood may provide evidence for the importance of CaR in integration of tissue responses.

Other $Ca^{2+}{}_o$ sensors in specialized environments

We have focused our discussion of the potential contributions of [$Ca^{2+}{}_o$] fluctuations on CaR because it represents the cleanest example of a Ca^{2+}-sensitive "sensor". It should be noted, however, that a variety of membrane-localized proteins sensitive to $Ca^{2+}{}_o$ fluctuations have

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been identified, including metabotropic glutamate receptors, particularly subtypes 1 and 5 (Saunders et al., 1998; Kubo et al., 1998, but see also Nash et al., 2001), $GABA_B$ receptors (Tabata et al., 2004), calcitonin receptors (Stroop et al., 1993), gp330/Megalin (Lundgren et al., 1997), TRPM7 channels (Wei et al., 2007), gap junction hemichannels (Stout & Charles, 2003), Notch (Rand et al., 2000; Raya et al., 2004), ASIC1a/ASIC1b channels (Babini et al., 2002), and a variety of voltage-gated cation channels (reviewed by Hofer, 2005). This partial list will likely expand as the importance of $[Ca^{2+}{}_o]$ fluctuations in modulating cell signaling is appreciated, and explicitly tested in a variety of experimental contexts.

Conclusions

Recent results suggest that $Ca^{2+}{}_o$ fluctuations occur as a natural consequence of agonist-activated cellular signaling which increases $Ca^{2+}{}_o$, primarily as a result of PMCA-mediated export of Ca^{2+} released from intracellular stores. The presence of CaR on the activated cell or adjacent cells initiates a wave of Ca^{2+} signaling through a multicellular network, not through cell-cell coupling via gap junctions but through the diffusion-restricted extracellular space. Other Ca^{2+}-sensitive sensors may assume similar integrative roles in a tissue-specific manner. Extracellular Ca^{2+} may represent the "tip of the iceberg" with respect to small molecules which might serve to signal cell activation and lead to cell network integration. cAMP (reviewed by Hofer & Lefkimmiatis, 2007), ATP (reviewed by Zimmermann, 2007), and cGMP (Sager, 2004) are exported from cells in physiologically significant amounts and may serve to integrate non-Ca^{2+} signaling pathways, although the receptors and functionally important niches have not in all cases been identified. It is clear that the interstitial environment represents a unique signaling compartment which can profoundly influence the behavior of cells in a tissue. Needed now are experimental approaches which permit the characterization and manipulation of this compartment *in vivo*.

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Abbreviations

CaR, Calcium sensing receptor; $Ca^{2+}{}_o$, extracellular calcium; GPCR, G protein-coupled receptor; $Ca^{2+}{}_i$, intracellular calcium; NCX, Na^+/Ca^{2+} exchanger; PTH, parathyroid hormone; PMCA, plasma membrane Ca^{2+} ATPase; $1,25(OH)_2D$, vitamin D.

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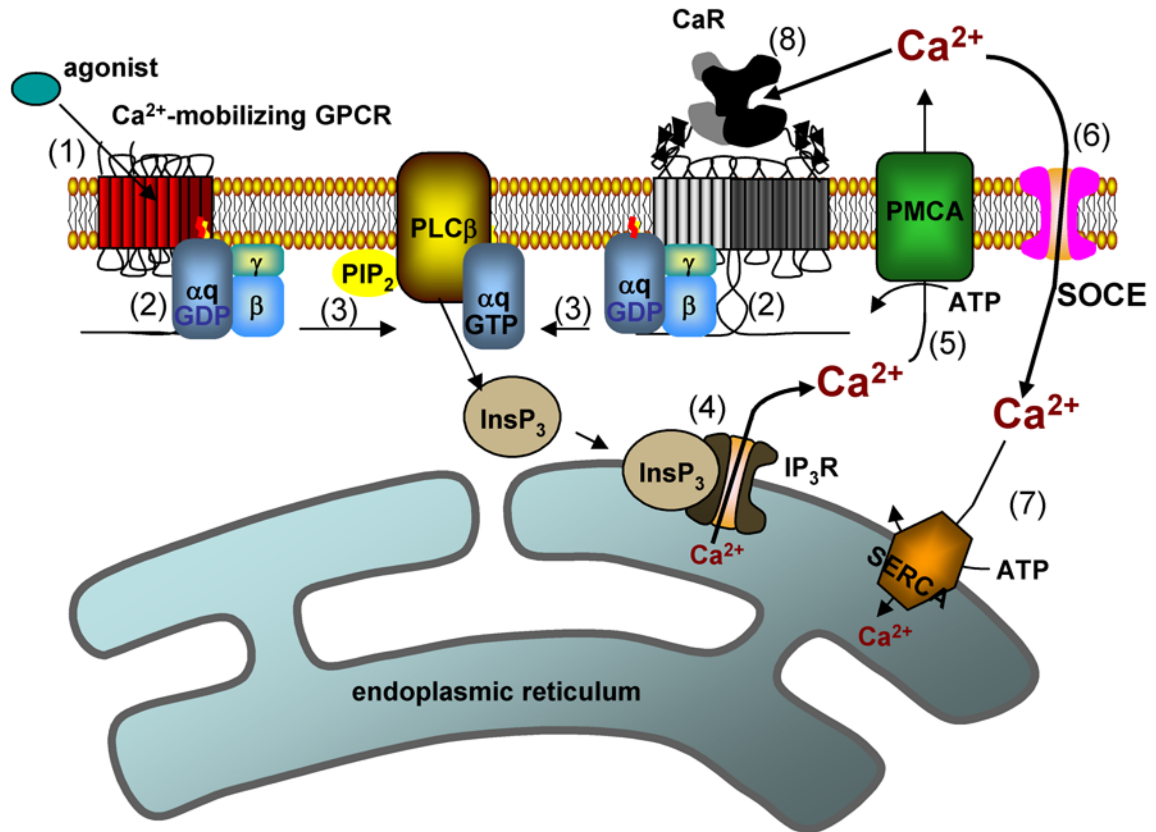

Figure 1. Molecular players in CaR-mediated autocrine/paracrine integration of Ca^{2+}-mediated signaling

Agonist activation of a Ca^{2+}-mobilizing GPCR (1) activates heterotrimeric G protein G_q (2), leading to activation of phospholipase Cβ (PLCβ)(3), and generation of inositol 1,4,5-trisphosphate ($InsP_3$), which binds to the endoplasmic reticulum-localized inositol trisphosphate receptor (IP_3R) (4), inducing release of Ca^{2+} into the cytosol. Most of the Ca^{2+} released from the endoplasmic reticulum is pumped out of the cell by the plasma membrane-localized Ca^{2+} ATPase (PMCA) (5). Restitution of endoplasmic reticulum Ca^{2+} content occurs via store-operated Ca^{2+} entry channels (SOCE) (6) and the sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA) (7). If the activated cell also expresses the calcium sensing receptor (CaR), potentiation of the response is possible, as PMCA pumps Ca^{2+} out of the cell into a restricted diffusion space, significantly increasing the $[Ca^{2+}{}_o]$, leading to CaR activation (8).

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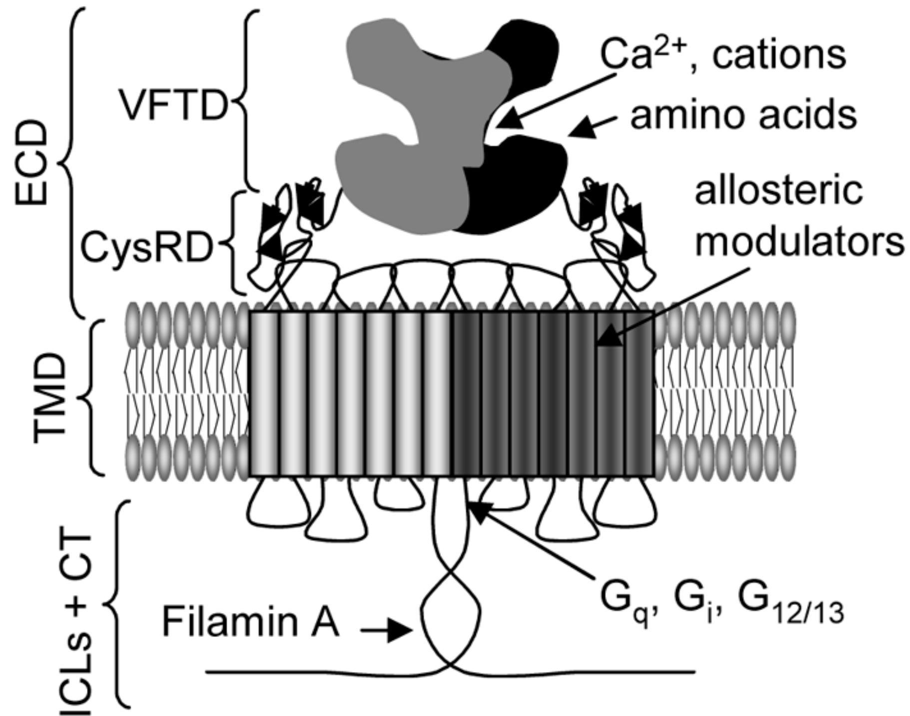

Figure 2. Domain structure of CaR

CaR has a large extracellular domain of >600 amino acid residues (ECD) consisting of a venus flytrap-like domain (VFTD) binding module with binding sites for Ca^{2+} and polyvalent cations and a distinct site for amino acids. The VFTD is coupled to the transmembrane heptahelical domain (TMD) by a cysteine-rich domain (CysRD) which is critical for coupling agonist binding to transmembrane domain activation. The TMD contains binding site(s) for positive and negative allosteric modulators of CaR. The intracellular loops (ICLs) plus large (215 amino acid residues) carboxyl terminus (CT) contain binding sites for heterotrimeric G proteins as well as the cytoskeletal scaffold protein Filamin A. CaR can also interact with RAMPs 1 and 3 (not shown). See text for details and references.

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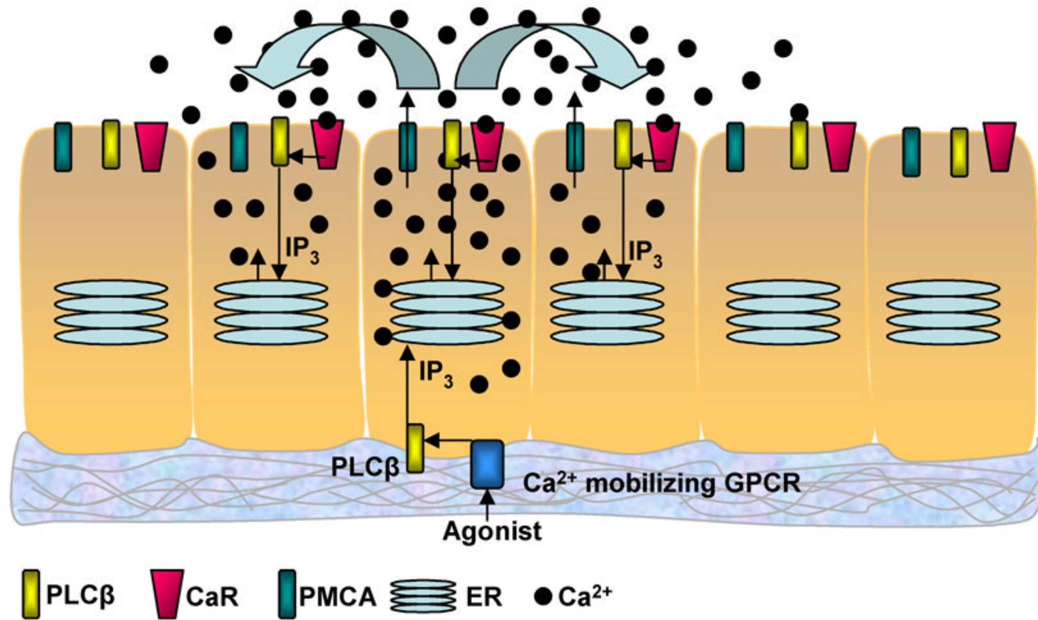

Figure 3. Potential mechanism for CaR-mediated integration of Ca2+ signaling

When a single cell (or a few cells) in a multicellular network is (are) activated by an agonist for a Ca^{2+}-mobilizing GPCR, $Ca^{2+}{}_{i}$ increases, and is pumped out of the cell via PMCA. The local increase in $[Ca^{2+}{}_{o}]$ in the restricted diffusion space surrounding the cells potentiates the activity in the agonist-activated cell (autocrine activation), and also activates CaR on adjacent cells (paracrine activation). CaR activation increases $Ca^{2+}{}_{i}$, leading to PMCA-mediated Ca^{2+} efflux in adjacent cells, propagating the Ca^{2+} signaling response through the tissue. In this example, CaR is present in proximity to PMCA, which has been observed in epithelial tissue (De Luisi & Hofer, 2003). For details of the signaling pathways, see Figure 1.