REVIEW / SYNTHÈSE

Function- and agonist-specific Ca^{2+} signalling: The requirement for and mechanism of spatial and temporal complexity in Ca^{2+} signals

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Abstract: Calcium signals have been implicated in the regulation of many diverse cellular processes. The problem of how information from extracellular signals is delivered with specificity and fidelity using fluctuations in cytosolic Ca^{2+} concentration remains unresolved. The capacity of cells to generate Ca^{2+} signals of sufficient spatial and temporal complexity is the primary constraint on their ability to effectively encode information through Ca^{2+}. Over the past decade, a large body of literature has dealt with some basic features of Ca^{2+}-handling in cells, as well as the multiplicity and functional diversity of intracellular Ca^{2+} stores and extracellular Ca^{2+} influx pathways. In principle, physiologists now have the necessary information to attack the problem of function- and agonist-specificity in Ca^{2+} signal transduction. This review explores the data indicating that Ca^{2+} release from diverse sources, including many types of intracellular stores, generates Ca^{2+} signals with sufficient complexity to regulate the vast number of cellular functions that have been reported as Ca^{2+}-dependent. Some examples where such complexity may relate to neuroendocrine regulation of hormone secretion/synthesis are discussed. We show that the functional and spatial heterogeneity of Ca^{2+} stores generates Ca^{2+} signals with sufficient spatiotemporal complexity to simultaneously control multiple Ca^{2+}-dependent cellular functions in neuroendocrine systems.

Key words: signal coding, IP_3 receptor, ryanodine receptor, endoplasmic reticulum, Golgi, secretory granules, mitochondria, exocytosis.

Résumé : Des signaux de calcium interviennent dans la régulation de plusieurs processus cellulaires variés. Le mécanisme par lequel des signaux extracellulaires sont transmis spécifiquement et fidèlement par des fluctuations de la concentration cytosolique du Ca^{2+} demeure inconnu. La capacité des cellules de produire des signaux de Ca^{2+} ayant une complexité spatiale et temporelle suffisante est la première contrainte pour pouvoir encoder efficacement une information par l'intermédiaire du Ca^{2+}. Au cours de la dernière décennie, de nombreux articles ont décrit les caractéristiques de base de la distribution du Ca^{2+} dans les cellules, ainsi que la multiplicité et la diversité fonctionnelle des réserves intracellulaires de Ca^{2+} et des voies d'entrée du Ca^{2+} à l'intérieur des cellules. En principe, les physiologistes ont maintenant l'information nécessaire pour s'attaquer au problème de la spécificité de fonction et d'agonistes des voies de transduction d'un signal par le Ca^{2+}. Cette revue porte sur les données indiquant que la libération de Ca^{2+} de diverses sources, tels plusieurs types de réserves intracellulaires, produit des signaux de Ca^{2+} ayant une complexité suffisante pour régler le très grand nombre de fonctions cellulaires qui seraient Ca^{2+}-dépendantes. Quelques exemples où

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Abbreviations: $[Ca^{2+}]_c$, cytosolic free calcium concentration; $[Ca^{2+}]_m$, mitochondrial free calcium concentration; $[Ca^{2+}]_L$, lumenal free calcium concentration; $[Ca^{2+}]_{SG}$, secretory granule calcium concentration; cADPR, cyclic adenosine diphosphate ribose; CaM, calmodulin; CaMK, Ca^{2+}/calmodulin-dependent protein kinase; cGnRH-II, chicken gonadotropin-releasing hormone-II; CICR, Ca^{2+}-induced Ca^{2+} release; DAG, 1,2-diacylglycerol; ER, endoplasmic reticulum; GFP, green fluorescent protein; IP_3, inositol 1,4,5-trisphosphate; IP_3R, inositol 1,4,5-trisphosphate receptor; NAADP, nicotinic acid-adenine dinucleotide phosphate; NCX, sodium-calcium exchange; NF-AT, nuclear factor of activated T cells; NF-κB, nuclear factor-κB; PACAP, pituitary adenylate cyclase-activating polypeptide; PMCA, plasma membrane Ca^{2+}-ATPase; PLC, phospholipase C; PLA_2, phospholipase A_2; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; RyR, ryanodine receptor; S1P, sphingosine 1-phosphate; SCaMPER, sphingolipid Ca^{2+} release-mediating protein of endoplasmic reticulum; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; sGnRH, salmon gonadotropin-releasing hormone; SOC, store-operated Ca^{2+} channels; SPC, sphingosylphosphorylcholine; TRP, transient receptor potential; VDCC, voltage-dependent Ca^{2+} channels.

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cette complexité interviendrait dans la régulation neuroendocrinienne de la synthèse et la sécrétion d'une hormone sont discutés. Nous montrons que l'hétérogénéité spatiale et fonctionnelle des réserves de Ca^{2+} engendre des signaux de Ca^{2+} ayant une complexité spatio-temporelle suffisante pour assurer la régulation simultanée de plusieurs fonctions cellulaires Ca^{2+}-dépendantes dans des systèmes neuroendocriniens.

Mots clés : codage d'un signal, récepteur de l'inositol triphosphate, récepteur de la ryanodine, réticulum endoplasmique, Golgi, granules de sécrétion, mitochondries, exocytose.

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Introduction

Ca^{2+} signalling is a ubiquitous, multifunctional, and conserved mechanism for the transduction of information in living cells (Clapham 1995; Petersen et al. 1994; Berridge 1997; Trewavas et al. 1996). Ca^{2+} signals can be defined as controlled deviations from the resting cytosolic Ca^{2+} ion concentration ($[Ca^{2+}]_c$). Since prolonged elevations in $[Ca^{2+}]_c$ are lethal (Clapham 1995; Carafoli 1987; Choi 1992) cells expend considerable energy maintaining very low cytosolic Ca^{2+} levels (typically 50–200 nM free Ca^{2+}) compared to the extracellular environment which is in the millimolar range (Clapham 1995; Carafoli 1987). This steep gradient, accomplished by expulsion of Ca^{2+} from the cytosol into the extracellular space and intracellular organelles, combined with the presence of Ca^{2+}-selective ion channels on the plasma and organelle membranes, allows Ca^{2+} signals to be generated rapidly.

The extensive use of Ca^{2+}-sensitive fluorescent probes, and more recently, targeted aequorin chimeras (Rizzuto et al. 1993) and green fluorescent protein (GFP)-based indicators (Miyawaki et al. 1999), have permitted a detailed understanding of the complexity of Ca^{2+} signalling. Meanwhile, the spatial and temporal resolution in measurements of various Ca^{2+}-dependent cellular functions, especially exocytosis, has also improved dramatically (Augustine and Neher 1992; Zhou and Misler 1996; Angles et al. 1999). Together, these advances are providing novel insights into how information, in the form of Ca^{2+} signals, can be targeted to specific cellular outcomes.

The purpose of this article is not to comprehensively review the entire literature of Ca^{2+} signalling. Instead, we shall endeavor to focus on recent biophysical, biochemical, and theoretical advances in the study of signal transduction involving intracellular Ca^{2+} signals. A limited number of examples, primarily from the biochemical and biophysical literature, will be used to illustrate the potential complexity of Ca^{2+} signalling. Another goal of this review is to provide insight as to how these fundamental data on the complexity of Ca^{2+} signalling can be applied to the study of neuroendocrine regulation. Unfortunately, studies that incorporate an appreciation for multiple Ca^{2+} stores and the diversity of Ca^{2+}-dependent target functions are rare at the physiological level. In many cases, only speculation on the physiological relevance is possible at this time. Nevertheless, we hope this article will catalyze research into the role of multiple Ca^{2+} stores and spatio-temporal Ca^{2+} signal complexity in the simultaneous regulation of distinct cellular functions in neuroendocrine systems.

This review is organized into three major sections. The first section will outline the problem. How does such a ubiquitous signalling molecule as Ca^{2+} ion relay information, with specificity, to a great variety of cellular functions? Second, we will examine the ultrastructural and molecular heterogeneity of Ca^{2+} sources, with special emphasis on multiple novel intracellular Ca^{2+} stores. Third, we will examine whether cells generate Ca^{2+} signals with sufficient complexity to control multiple cellular functions, by looking at examples of function- and agonist-specific Ca^{2+} signalling in a few selected neuroendocrine systems.

Section I: Multiple Ca^{2+}-dependent signalling cascades and cellular functions

Diversity of Ca^{2+}-dependent cell functions

Individual cells generate multiple Ca^{2+}-modulated outcomes through the selective regulation of a bewildering number of Ca^{2+}-regulated enzymes. The spectrum of Ca^{2+} involvement ranges from permissive to triggering actions. One ubiquitous and conserved enzyme that has received particular attention is calmodulin (CaM), which binds Ca^{2+} through conserved motifs known as EF hands (Weinstein and Mehler 1994). CaM is probably the major physiologically active Ca^{2+} binding protein in most cells (Braun and Schulman 1995). Recent evidence suggests that CaM can faithfully transduce the spatial and temporal complexity of Ca^{2+} signals (Inagaki et al. 1997; Craske et al. 1999). CaM can regulate the activity of many of enzymes. Among the targets for activation CaM are components of other signal transduction cascades, including membrane ion channels (Levitan 1999; Braun and Schulman 1995).

Many researchers have focused on pathways regulated by Ca^{2+}-dependent kinases, such as CaMkinases (CaMK), and phosphatases, such as calcineurin (Braun and Schulman 1995; Yakel 1997; Westphal et al. 1998). Once CaMK is activated, it prolongs the transduction of the information encoded by the Ca^{2+} signal, even after $[Ca^{2+}]_c$ has returned to basal levels (MacNicol et al. 1990). Ca^{2+}-dependent effects on cellular enzymes can be mediated either through direct binding of Ca^{2+} or through several classes of Ca^{2+} effector proteins, many of which contain either EF-hand motifs, as in CaM, or C2 domains such as those found in protein kinase C (PKC). Several key steps in other signal transduction pathways can also be modulated by Ca^{2+}, including plasma membrane ion channels and transporters (Levitan 1999; Tsien and Tsien 1990), conventional PKCs (α, βI, βII, and γ isoforms; Newton 1997), adenylate cyclases (Mons et al. 1998), phospholipase A_2 (PLA_2; Kramer and Sharp 1997), phospholipase C (PLC; Singer et al. 1997), phosphodiesterases (Braun and Schulman 1995), nitric oxide synthase (Griffith and Stuehr 1995), mitogen-activated protein kinase cascades (Egea et al. 1999), and the protease,

calpain (Saido et al. 1994). The physiological roles of several other classes of Ca^{2+}-binding proteins, including the S100 family (Schäfer and Heizmann 1996) and the annexin family (Burgoyne and Geisow 1989) are less well understood.

Although the list of signal transduction pathways presented above contains only a limited number of examples, it indicates clearly that Ca^{2+}-regulated signal transduction cascades may exert indirect, as well as direct, control over many cellular functions. The Ca^{2+} signalling cascades that participate in excitation-secretion coupling and excitation-contraction coupling are perhaps the most extensively studied (Neher 1998; Kasai 1999; Somlyo and Somlyo 1994; Braun and Schulman 1995). Ca^{2+} also plays important roles throughout the lifetime of cells/organisms, from fertilization (Gilkey et al. 1978) to death, whether it occurs through apoptosis, necrosis, or excitotoxicity (Trump and Berezesky 1996; Marks 1997; Kruman and Mattson 1999; Choi 1992). A role for Ca^{2+} signalling has been suggested in various parts of the cell cycle (Hepler 1994; Groigno and Whitaker 1998) and in differentiation (Gu and Spitzer 1995). Ca^{2+} has also been shown to be involved in cell adhesion and motility, cytoskeletal reorganization (Takeichi 1990; Witman 1993; Maxfield 1993; Pettit and Fay 1998; Janmey 1994) and restructuring/repositioning of organelles, including Ca^{2+} stores such as the endoplasmic reticulum (ER; Subramanian and Meyer 1997). Ca^{2+} is also an important intercellular signal, mediating cell-to-cell signalling through the actions of gap junctions (Sanderson 1996) or Ca^{2+}-sensing receptors, which are widely distributed (Shorte and Schofield 1996; Nemeth 1995; Chattopadhyay et al. 1996).

Cellular metabolism may also be linked to the regulation of Ca^{2+}-sensitive mitochondrial dehydrogenases (Hajnóczky et al. 1995), and possibly ATP synthase (Hubbard and McHugh 1996). Hormonal stimulation of hepatocytes with vasopressin evokes cytosolic Ca^{2+} signals, which in turn regulate mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_m$). Temporally resolved measurements of $[Ca^{2+}]_c$, $[Ca^{2+}]_m$, and the redox state of pyridine nucleotides, have demonstrated that cellular metabolism is regulated by the frequency of Ca^{2+} oscillations (Hajnóczky et al. 1995; Robb-Gaspers et al. 1998). Similar techniques were used to demonstrate that pancreatic β-cell metabolism is also controlled by Ca^{2+} oscillations. The regulation of cellular metabolic rate by Ca^{2+} signals is probably a general mechanism of ensuring that energy metabolism is up-regulated in activated cells (Pralong et al. 1994; Duchen 1999).

In some cases, Ca^{2+} participates in opposite elements of the same physiological function. For example, in addition to triggering contraction, high-amplitude, localized Ca^{2+} release events from intracellular channels activate Ca^{2+}-activated potassium channels leading to relaxation in smooth muscle (Nelson et al. 1995). In the brain, Ca^{2+} plays an important role in both long-term potentiation and long-term depression of synaptic transmission (Svoboda and Mainen 1999). Ca^{2+} affects several components of the secretory pathway, including both exocytosis and endocytosis (Klingauf et al. 1998; De Camilli and Takei 1996; Lai et al. 1999). Many parts of the secretory pathways can be controlled independently, and thus require specific Ca^{2+} signals. This article will devote considerable attention to this issue, since it is of particular interest to neuroendocrinologists.

Ca^{2+} regulation of multiple sites on the protein synthesis/secretory pathway

Ca^{2+} is known to play a role in several elements of the extended secretory pathway, which includes hormone gene expression and synthesis. Many aspects of this pathway can be modulated independently, often as a consequence of hormonal control. Ca^{2+}-dependent gene expression can be regulated by a great number of positive and negative regulators of transcription, including the cyclic AMP response element-binding protein, serum response factor, c-Jun and many others (Shaywitz and Greenberg 1999; Cruzalegui et al. 1999; Johnson et al. 1997; Roche and Prentki 1994). These transcription factors integrate other signal transduction cascades with Ca^{2+}-dependent signalling (Shaywitz and Greenberg 1999). The ubiquitous and pleiotropic transcription factor nuclear factor-κB (NF-κB; F. Chen et al. 1999) is a component of one of the best understood Ca^{2+} signalling pathways. As with NF-κB, studies on nuclear factor of activated T cells (NF-AT), which is regulated by calcineurin, have provided insight into the regulation of multiple targets by Ca^{2+} signals (Crabtree 1999). Stimulatory transcription factors are thought to be regulated indirectly by Ca^{2+}-activated kinase pathways (Bading et al. 1993). In contrast, Ca^{2+} binds directly to EF-hand motifs on the transcriptional repressor known as the downstream regulatory element antagonist modulator, and releases it from the downstream regulatory element, thereby derepressing the target gene (Carrion et al. 1999). Curiously, Ca^{2+} may modulate the transcriptional activity of members of the nuclear hormone receptor superfamily through calreticulin, a Ca^{2+}-binding lectin chaperone located in the ER (Burns et al. 1994; Perrone et al. 1999; Krause and Michalak 1997). Taken together, these reports demonstrate that Ca^{2+} can act through many mechanisms to control gene expression. Whether these activities are widespread in neuroendocrine systems remains to be tested.

Ca^{2+} has been implicated in the control of protein synthesis. For example, CaMK III regulates protein synthesis via phosphorylation of elongation factor-2 (Braun and Schulman 1995 and references therein). Ca^{2+} may also play a role in directly modulating the rate of initiation of protein translation (Brostrom and Brostom 1998). Specifically, depletion of Ca^{2+} from the ER, and not the elevation of $[Ca^{2+}]_c$, inhibits the rate of protein synthesis (Kimball and Jefferson 1992). In a variety of cell types, pharmacological or hormonal depletion of Ca^{2+} stores impedes protein processing in the ER. This event initiates a cascade of signals, culminating in the phosphorylation of eukaryotic initiation factor 2α, which slows the rate of translational initiation (Weiler et al. 1996; Reilly et al. 1998; reviewed in Brostrom and Brostom 1998). Recently, Biswas and co-workers (1999) proposed that mitochondrial stress signals are transduced to the nucleus via Ca^{2+} signals, a mechanism that is reminiscent of the transduction of ER stress signals to the nucleus (Pahl and Baeuerle 1997).

Farther down the secretory path, protein sorting, transport and packaging are also influenced by Ca^{2+} (particularly lumenal Ca^{2+}, $[Ca^{2+}]_L$). Studies have shown that the exit of

newly folded proteins from the ER (Lodish and Kong 1990) and their transport between the ER and Golgi (Beckers and Balch 1989) are inhibited by treatments that discharge ER Ca^{2+} stores. Intact Ca^{2+} homeostasis in the ER and Golgi is required for post-translational processing, including the proteolytic cleavage of many hormone precursors such as proinsulin and prosomatostatin (Di Jeso et al. 1997; Guest et al. 1997; Austin and Shields 1996). Some of the Ca^{2+}-regulated protein processing and folding may be mediated through ER lumenal proteins, such as calreticulin and an immunoglobulin binding protein known as BiP, that do double-duty as chaperone proteins and high-capacity Ca^{2+} buffering proteins (Krause and Michalak 1997; Corbett et al. 1999; Lièvremont et al. 1997). Decreasing $[Ca^{2+}]_L$ may also accelerate protein degradation (Wileman et al. 1991), and cause the loss of resident ER proteins (Booth and Koch 1989). Elevated $[Ca^{2+}]_L$ and acidic pH are required for sorting hormones and their processing enzymes into secretory granules of the regulated secretory pathway (Canaff et al. 1996; Song and Fricker 1995). It is possible that certain hormones may differentially aggregate at specific Ca^{2+} concentrations. If this were found to be typical, it would suggest that local Ca^{2+} gradients in the trans Golgi compartments may facilitate preferential sorting of hormones into specific granule populations. In neuroendocrine cells that release multiple peptide hormones, this may be of particular importance. For example, differential sorting has been reported for luteinizing hormone and follicle-stimulating hormone, which are known to be independently regulated during some parts of the rat reproductive cycle (Thomas and Clarke 1997; Childs et al. 1987).

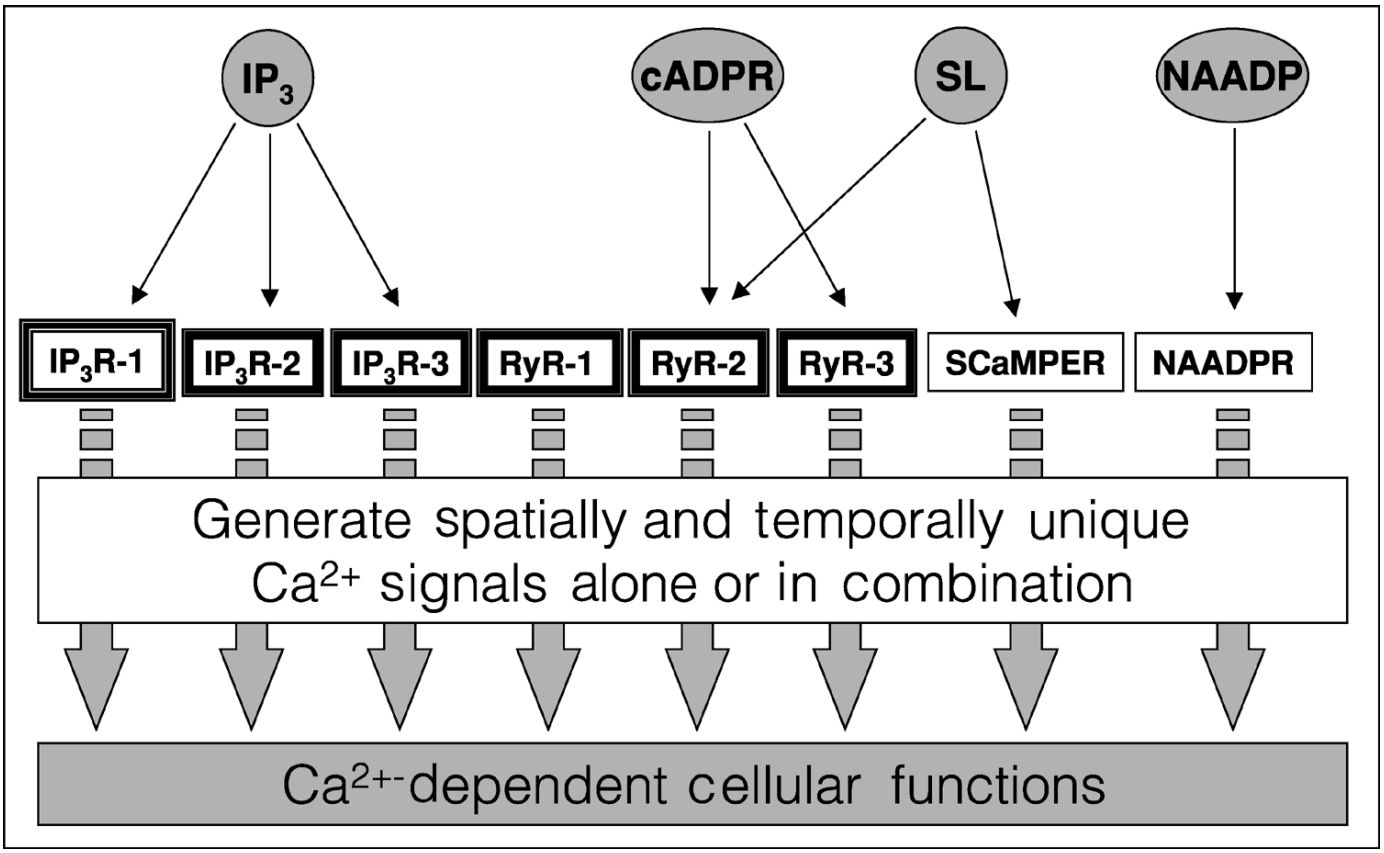
Prior to exocytosis, several reactions such as granule transport, docking and priming may also be regulated by Ca^{2+} in several cell types (Neher and Zucker 1993; Smith et al. 1998; Y.A. Chen et al. 1999). It is generally thought that these reactions occur prior to, and do not obligate, the final step of membrane fusion. In this case, moderate Ca^{2+} elevations may modulate priming, while exocytosis may require higher local $[Ca^{2+}]$ (Neher 1998; Y.A. Chen et al. 1999). Therefore, it is likely that these steps in the secretory pathway are specifically modulated by the spatial segregation and amplitude of Ca^{2+} signals.

Several novel developments in the study of Ca^{2+}-triggered exocytosis have recently been reported. A general scheme for the control of exocytosis by localized micromolar Ca^{2+} domains has been widely accepted and these fundamental considerations have been reviewed elsewhere (Kasai 1999; Neher 1998; Südhof 1995). One new area of interest centers around the possible differential involvement of Ca^{2+} in several distinct modes of secretion. Although it is clear that Ca^{2+} signals trigger agonist-evoked exocytosis, much less is known about the involvement of Ca^{2+} in the basal secretion that is prominent in many neuroendocrine cell types, and may be controlled independently (see, for example, Chang et al. this issue). Basal release can arise from the constitutive or constitutive-like secretory pathways or a steady leak from a pool secretory granules in the regulated pathway (Burgess and Kelly 1987; Kuliawat and Arvan 1992). Although a dramatic elevation of $[Ca^{2+}]_c$ is not required for basal exocytosis in some neuroendocrine systems (Masumoto et al. 1995; J.D. Johnson and J.P. Chang, unpublished), Ca^{2+} levels in the lumen of secretory granules ($[Ca^{2+}]_{SG}$) could conceivably play a role in hormone release under unstimulated conditions. Indeed, constitutive homotypic membrane fusion of yeast vacuole, is mediated by Ca^{2+} released from internal stores acting through CaM (Peters and Mayer 1998). CaM and PKC have been shown to enhance the sensitivity of the secretory apparatus to existing Ca^{2+} levels (Y.A. Chen et al. 1999). These data form the basis for the hypothesis that basal secretion in neuroendocrine systems may result from secretory granules that carry with them sufficient Ca^{2+} which, when released in a highly localized manner, promotes their exocytosis. Such a basal release pathway could be independent of large-scale changes in $[Ca^{2+}]_c$ and could, theoretically, be modulated by agents that reduce basal hormone release.

Another very interesting and novel topic is the post-fusion regulation of exocytosis in the regulated secretory pathway (Rahaminoff and Fernandez 1997). Evidence from morphological, electrophysiological and amperometry studies indicate that secretory material can be released without complete fusion of the secretory granule in a variety of cell types, including chromaffin cells and pancreatic β-cells (Rahaminoff and Fernandez 1997; Zhou and Misler 1996). This ultra-fast secretory granule recycling process bypasses the classical, clathrin-mediated endocytosis pathway. The probability of this so called kiss-and-run exocytosis increases with high Ca^{2+}, and can be modulated by hormones (Alés et al. 1999; Angelson et al. 1999). In addition to increasing the frequency of this kiss-and-run exocytosis, Ca^{2+} has the additional effect of alkalinizing the lumen of the secretory granules, thereby facilitating the leak of peptide contents into the extracellular space through an open, or partially open, fusion pore (Han et al. 1999). Taken together, these results also suggest that regulation of $[Ca^{2+}]_c$ or $[Ca^{2+}]_{SG}$ may by a target for treatments that inhibit basal hormone release. These findings likely represent only a first glimpse at numerous novel and physiologically important mechanisms for regulating hormone and transmitter release.

Current evidence strongly indicates that Ca^{2+} is involved in most facets of the secretory pathway, from gene transcription to post-fusion regulation of exocytosis. It is apparent that the secretory pathway is exquisitely sensitive to alterations in Ca^{2+} homeostasis, especially in the lumenal compartments. At present, many of the findings concerning Ca^{2+} regulation of different parts of the extended secretory pathway are difficult to reconcile. For example, further work is required to reconcile the dilemma that gene expression and exocytosis stimulated by many agonists seem to deplete intracellular Ca^{2+} stores, a maneuver that may generally result in a reduction of translational initiation and protein processing. There is an urgent need for studies that measure multiple Ca^{2+}-dependent cellular functions, as well as the Ca^{2+} signals in multiple compartments/subcompartments, all in spatial and temporal detail. Depending on what these studies find, there may be a need to examine the problem in the context of multiple function-specific Ca^{2+} stores (see Section II). The use of well-characterized physiological agonists will also be important, since agents like ionomycin and thapsigargin may simultaneously deplete several classes of Ca^{2+} stores, while preventing the normal sequestration and cycling of Ca^{2+}. Manipulations that mimic the unique

Fig. 1. Potential functional heterogeneity of Ca^{2+} stores in neuroendocrine cells. Several Ca^{2+} release channels, and the second messenger cascades that regulate them, are shown. Each of these Ca^{2+} channel types, or isoforms, may generate Ca^{2+} signals with different spatial and temporal characteristics. Ca^{2+}-release proteins that are positively regulated by Ca^{2+} at most physiological [Ca^{2+}] have a double border. Triple border indicates both activation and inactivation by Ca^{2+} in the physiological [Ca^{2+}] range. Individual components shown interact in cases of Ca^{2+} regulation. Addition functional diversity that may arise from putative IP_3R and RyR heterotetramers is not shown. Abbreviations: IP_3, inositol 1,4,5 trisphosphate; IP_3R, IP_3 Ca^{2+}-release channel; cADPR, cyclic adenosine 5′-diphosphate ribose; RyR, ryanodine receptor Ca^{2+}-release channel; SL, Ca^{2+}-mobilizing sphingolipids; SCaMPER, sphingolipid Ca^{2+}-release-mediating protein of endoplasmic reticulum; NAADP, nicotinic acid dinucleotide phosphate; NAADPR, putative NAADP Ca^{2+}-release channel.



spatial and temporal features of agonist-evoked Ca^{2+} signals are needed, ones which circumvent the potential pitfalls associated with non-selective Ca^{2+} drugs. For example, Ca^{2+} oscillations common to many neuroendocrine regulators may strike a balance, in time and space, between the alternative needs for both Ca^{2+} required for protein processing, and Ca^{2+} needed to trigger exocytosis.

Nevertheless, it is clear that very complex Ca^{2+} signalling strategies are required to specifically regulate different elements of the extended secretory pathway, which can be either functionally coupled or independent of each other. This problem is confounded by the numerous other Ca^{2+}-sensitive activities that progress simultaneously in living cells. The obligatory complexity and specificity in Ca^{2+} signalling may be imparted through strategies that involve the spatial and (or) temporal coding of Ca^{2+} signals made possible by the heterogeneity of Ca^{2+} stores and influx pathways found in many cell types.

Section II: Heterogeneity of intracellular Ca^{2+} stores and influx pathways

One possible solution to the problem of selective control over multiple Ca^{2+}-regulated cellular processes is to have multiple Ca^{2+} sources, each with distinct Ca^{2+} release and uptake dynamics, that can act alone or in combination to code for specific outcomes (Fig. 1). This section will explore this possibility. Relatively little is known about the contributions of multiple Ca^{2+} stores to the regulation of multiple cellular functions. In the past, researchers considered only one type of intracellular Ca^{2+} store in most cell types. However, multiple distinct intracellular Ca^{2+} stores are generally found in systematic studies, including, but limited to, the ER, nucleus, mitochondria, Golgi, secretory granules, and the cytosol proper (Pozzan et al. 1994; Pezzati et al. 1997; Tanaka and Tashjian 1993)(Fig. 2). At least five pharmacologically distinct Ca^{2+} pools have been assigned to the ER alone, if one considers evidence across cell types (Fig. 1). It

is likely that multiple distinct subcompartments with unique functional properties also exist within the other Ca^{2+}-storing organelles.

The cytosol as a determinant of Ca^{2+} signalling

One of the most important, and often overlooked, Ca^{2+} signalling components in the cell is the cytosol proper. The cytosol has remarkable Ca^{2+} buffering properties, and is the prime determinant of the spatial and temporal characteristics of Ca^{2+} signals (Allbritton et al. 1992; Clapham 1995; See Section III). Generally, free Ca^{2+} accounts for less than 1% of the total cytosolic concentration, although some variability exists in the values obtained from different cell types (Tse et al. 1994; Neher and Augustine 1992; Clapham 1995; Carafoli 1987). It is interesting to consider the possibility that cytosolic Ca^{2+} buffering capacity may be regulated hormonally. It is also likely that subtle differences exist in the cytosolic buffering properties between cell types. Notwithstanding, its is clear that the ability of cells to maintain a low resting $[Ca^{2+}]_c$ is critical for survival, as well as signalling.

Ca^{2+} can be pumped out of the cytosol by several mechanisms, including a multi-gene family of plasma membrane Ca^{2+}ATPases (PMCA), and Na^+/Ca^{2+} exchange (NCX; Carafoli 1992). The relative importance of PMCA and NCX is cell-type-dependent (Monteith and Roufogalis 1995). Although active under basal conditions, the Ca^{2+} efflux mechanisms can be regulated by agonists, through many endogenous effectors (Monteith and Roufogalis 1995). The PMCA is stimulated directly by CaM, as well as phosphorylation by PKA or PKC (Carafoli 1992; Monteith and Roufogalis 1995).

Multiple extracellular Ca^{2+} influx pathways and their effects on intracellular Ca^{2+} stores

Cells harness the massive gradient of free Ca^{2+} between the cytosol and the extracellular space to generate fast and discrete signals. Influx of extracellular Ca^{2+} is mediated by a suite of voltage-dependent and -independent channels (reviewed in Tsien and Tsien 1990). Of these, voltage-dependent Ca^{2+} channels (VDCC) are best understood. Several classes of VDCC may contribute to agonist- and function-specific Ca^{2+} signals. How plasma membrane oscillators lead to regular fluctuations in $[Ca^{2+}]_c$ has received considerable experimental and theoretical attention. A thorough treatment of their underlying mechanisms is beyond the scope of this article. The reader is directed to reviews on these topics for details (Sneyd et al. 1995; Petersen et al. 1994; Fewtrell 1993).

Extracellular Ca^{2+} influx and efflux from intracellular stores are functionally intertwined in many ways. Ca^{2+} influx is required to refill agonist-sensitive Ca^{2+} stores in excitable and non-excitable cells. In most neuroendocrine cells studied to date, pharmacological or hormonal depletion of inositol 1,4,5-trisphosphate (IP_3), or caffeine-sensitive intracellular Ca^{2+} stores, leads to the activation of an inward cation current (Ca^{2+} specific in most cell types) through a group of channels known collectively as store-operated Ca^{2+} channels (SOC) that remain only partially characterized (Putney and McKay 1999; Bennett et al. 1998; Fomina and Nowycky 1999). The major candidates for the SOC include gene products from a family of homologues of the *Drosophila* transient receptor potential (*trp* and *trp*-like) channels that mediate phototransduction (designated TRP1 to TRP7; Xu et al. 1997; Vannier et al. 1999; Montell 1997; Putney 1999*a*; Putney 1999*b*). Given that the electrophysiological characteristics of native and recombinant TRP channels do not entirely match those of the best-characterized store-dependent conductances (namely I_{CRAC} in macrophages and lymphocytes), the involvement of additional proteins cannot be ruled out (Putney and McKay 1999).

The mechanism by which the stores transmit their "empty" signal to the plasma membrane is controversial (Clapham 1995; Putney and McKay 1999). Two ideas, the involvement of a Ca^{2+}-influx factor generated at the ER and the direct physical interaction of ER Ca^{2+}-release channels with store-operated channels, have received the most attention. Several recent studies have lent support to the latter proposal (reviewed in Putney 1999*a*, 1999*b*). For example, in TRP3-transfected HEK293 cells, store-activated single channel Ca^{2+} conductances were abolished in excised patches, but could be restored after a short wash with the addition of IP_3; after a more thorough wash, the IP_3 receptor (IP_3R) was also required to reconstitute TRP function (Kiselyov et al. 1998). These data, combined with evidence of protein-protein interactions between IP_3R and TRP3 (Kiselyov et al. 1998; Boulay et al. 1999; Putney 1999*a*) indicate the importance of physical coupling between one type of plasma membrane store-operated Ca^{2+} channel, and one type of ER Ca^{2+}-release channel. Although this likely represents an important mechanism, it cannot yet be considered universal, as it does not address how signals could be transmitted from empty Ca^{2+} stores that do not possess IP_3R (such as ryanodine-sensitive stores; Bennet et al. 1998) or that are physically distant from the plasma membrane. Furthermore, *trp* currents are unaffected in *Drosophila* photoreceptor cells engineered to lack IP_3Rs (Acharya et al. 1997), indicating that this mechanism for activating TRP channels may be absent in some native systems. Although several major advances in this area have been achieved, much more work is required before the complexity and diversity of store-operated Ca^{2+} pathways can be understood.

Regardless of the influx mechanism, it is clear that intracellular stores are highly dependent on extracellular Ca^{2+} availability. Exposure of cells to nominally Ca^{2+}-free conditions leads to the abrupt depletion (within several minutes) of rapidly exchanging intracellular stores (Tse et al. 1993). Thus, many conclusions that excluded the involvement of intracellular Ca^{2+} stores, based solely on the requirement of extracellular Ca^{2+}, may need to be revisited. It is obvious that Ca^{2+} entry through VDCC or SOC plays a vital permissive, and often paramount role, in cellular functioning, especially in certain cell types. The regulation of $[Ca^{2+}]_c$ by VDCC and other influx mechanisms has been thoroughly reviewed elsewhere (Berridge 1998; Tsien and Tsien 1990). Intracellular Ca^{2+} stores are ubiquitous and have been found to participate in a great number and variety of cell functions (Pozzan et al. 1994). The following subsections of this article will focus primarily on the role of Ca^{2+} released from organelles such as the ER, nucleus, mitochondria, Golgi, and secretory granules, and how these release processes may function at the molecular level.

The endoplasmic reticulum as a prototypical heterogeneous Ca^{2+} store

The importance of intracellular stores in Ca^{2+} signalling and basal Ca^{2+} homeostasis in now recognized for both excitable and non-excitable cells (Clapham 1995; Berridge 1998; Svoboda and Mainen 1999). Chief among intracellular Ca^{2+} stores, in terms of proposed physiological importance, is the sarco/endoplasmic reticulum (ER). The general principles of ER-mediated Ca^{2+} signalling have been reviewed elsewhere (Pozzan 1994; Tsien and Tsien 1990; Clapham 1995). In this article, we will focus on recent advances and concepts that are of particular importance to physiologists and neuroendocrinologists.

The ER Ca^{2+} concentration has been estimated to be in the millimolar range, using targeted aequorin (Montero et al. 1997), compartmentalized Ca^{2+} indicator dyes (Golovina and Blaustein 1997), and electron energy loss imaging (Pezzati et al. 1997). One of the most significant findings of these studies is that the Ca^{2+} levels in the ER were found to be heterogeneous. For example, Golovina and Blaustein (1997) imaged spatially and pharmacologically distinct ER Ca^{2+} stores in intact astrocytes and myocytes. Similarly, intact PC12 and HeLa cells possess both high [Ca^{2+}] and low [Ca^{2+}] ER subcompartments (Montero et al. 1997; Pezzati et al. 1997). The ER fractions of neuroendocrine PC12 cells also display considerable heterogeneity in the distribution of Ca^{2+} release channels, Ca^{2+} pump isoforms, and Ca^{2+} buffering proteins (Rooney and Meldolesi 1996). Taken together, these reports suggest that spatial, pharmacological, and functional heterogeneity may be a common feature of the ER in all cell types.

The most common intracellular Ca^{2+}-sequestering mechanisms are ATP-dependent transporters. The ubiquitous sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) family of genes encode five SERCA protein isoforms with different functional properties. The constitutive activity of SERCA is a major factor in cytoplasmic and ER Ca^{2+} homeostasis. All known SERCA isoforms are sensitive to inhibition by thapsigargin (Lytton et al. 1991). The activity of SERCA can be modulated by hormones, including insulin (Xu et al. 1999). Additional forms of ATP-dependent, thapsigargin-resistant Ca^{2+} transport may exist on a number of Ca^{2+}-storing organelles, including ER (see below; Pizzo et al. 1997; Tanaka and Tashjian 1993).

As is the case in cytosol, the lumen of the ER contains abundant Ca^{2+} buffering proteins. For example, calsequestrin and calreticulin, in muscle and non-muscle cells, respectively (Kawasaki and Kasai 1994; Krause and Michalak 1997), reduce the effective Ca^{2+} gradient that SERCAs work against. These proteins are important for many aspects of Ca^{2+} homeostasis in the lumen of the ER and other Ca^{2+} stores, and thus may affect Ca^{2+} signalling (Llewelyn et al. 1998; Krause and Michalak 1997). Overexpression of calreticulin has been shown to inhibit agonist-stimulated Ca^{2+} signals in *Xenopus* oocytes (Camacho and Lechleiter 1995). It has also been suggested that calreticulin may directly modulate the activity of SERCA (John et al. 1998). Taken together, these studies demonstrate that lumenal Ca^{2+} bindings proteins can have diverse roles in cell functioning, aside from the accumulation of Ca^{2+} for cytosolic signals.

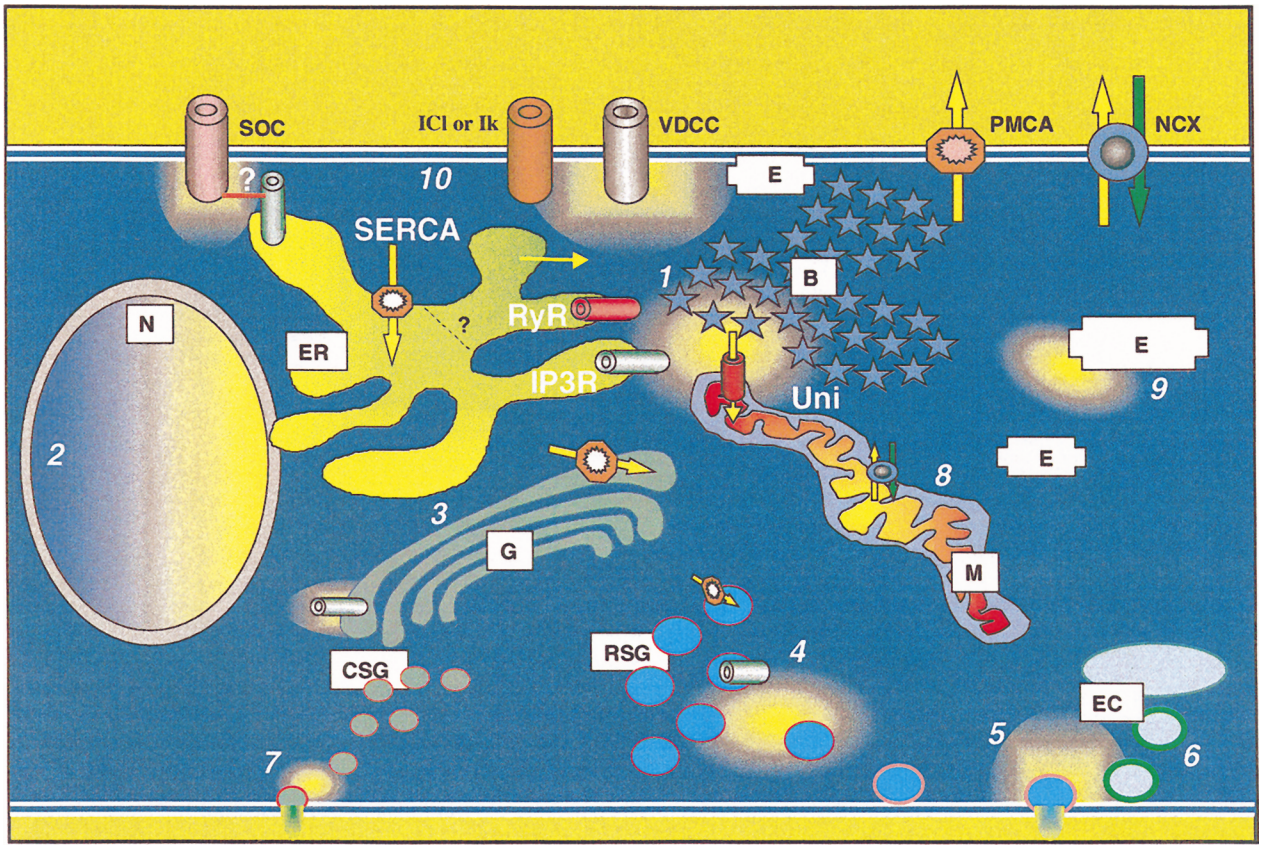
Two types of widely expressed ER Ca^{2+}-release channel have been studied in detail. Both the IP_3R and the ryanodine receptor (RyR) belong to families encoded by three known genes, although additional isoforms of both proteins are produced by alternative splicing (Pozzan et al. 1994; Phillips et al. 1996). The IP_3R genes and RyR genes encode related products that form large highly conserved tetramers (300 and 550 kDA; Berridge 1997; Pozzan et al. 1994). The most important functional commonality is that, both the IP_3R and RyR Ca^{2+} channels are opened by Ca^{2+}; their respective second messengers, IP_3 and cADPribose, modulate the Ca^{2+}-activation threshold (Bezprozvanny et al. 1991; Berridge 1997). IP_3 activates its receptor channel by relieving the inhibitory effects of high Ca^{2+} (Mak et al. 1998). Although the IP_3R and RyR proteins are functionally similar, there are important kinetic differences may which underlie the diversity of Ca^{2+} signals generated by these channels. For example, the IP_3R and the RyR differ with respect to their conductance, with the latter being greater (Bezprozvanny et al. 1991). Although these two Ca^{2+}-mobilizing pathways share many common features, the functional differences discussed below may provide the basis for function- and agonist-specific Ca^{2+} signalling.

The IP_3 receptor signalling system

The signalling cascade initiated by agonist activation of heterotrimeric GTP-binding protein ($G_{q/11}$) stimulation of phospholipase C (PLCβ), resulting in the hydrolysis of phosphatidylinositol 4,5-bisphosphate to IP_3 and 1,2-diacylglycerol (DAG), has been thoroughly studied in many cell types (Clapham 1995; Petersen et al. 1994; Berridge 1987). This reaction represents a major bifurcation in the signal transduction of extracellular regulators, since DAG is an endogenous activator of PKC (Berridge 1987). IP_3 is a highly diffusible second messenger that binds to the IP_3R Ca^{2+} channel on intracellular membranes. IP_3 is metabolized by trisphosphatase into inositol 1,4-bisphosphate, which does not have Ca^{2+}-releasing properties (Berridge 1987). Although IP_3 metabolism also results in the formation of other inositol phosphates, which are thought be physiologically important, the actions of IP_3 are by far the best understood.

Three sub-types of the IP_3R have been characterized. The relative abundance of these proteins is controlled in a developmentally regulated and tissue-specific manner, suggesting distinct physiological roles for each subtype, or combination thereof (Miyakawa et al. 1999; Wojcikiewicz 1995). Accordingly, the known IP_3R isoforms display important functional differences. Individual IP_3R subtypes, and possibly other heterotetrameric isoforms, differ with respect to their Ca^{2+} and ATP sensitivity (Miyakawa et al. 1999; Ramos-Franco et al. 1998). Based on single-channel studies of the IP_3R-1 in lipid bilayers, the IP_3R channel was thought to have a "bell-shaped" sensitivity to [Ca^{2+}], which peaks with maximal stimulation at ~300 nM Ca^{2+} (Bezprozvanny et al. 1991; Mak et al. 1998). In contrast, the IP_3R-2 and IP_3R-3 are not inhibited by high [Ca^{2+}] (Hagar et al. 1998; Ramos-Franco et al. 1998). These findings suggest that the IP_3R-2 and IP_3R-3 release more Ca^{2+} in vivo, because their activity is not self-limiting. Experimental evidence suggests that the IP_3R-3 mediates the initial amplification step that triggers

Fig. 2. Possible roles of localized Ca^{2+} signals in neuroendocrine cells. High [Ca^{2+}] is depicted in yellow. The white numbers correspond to the following Ca^{2+}-dependent cellular functions that may be regulated by localized Ca^{2+}signals. Local Ca^{2+} events, constricted by the presence of Ca^{2+} buffers, are recruited in the generation of global Ca^{2+} signals (1). The integration and resolution of Ca^{2+} signals coded in frequency, duration, and amplitude in the nucleus can regulate the expression of specific genes (2). Localized high-Ca^{2+} environments are required for protein production and processing in the ER and Golgi (3). Ca^{2+} is required for transport and docking of secretory granules (4). Local high-amplitude Ca^{2+} domains trigger exocytosis (5) and may play a role in endocytosis (6). Since it is required for constitutive membrane fusion, localized Ca^{2+} elevations may also mediate exocytosis in the constitutive secretory pathway (7). Ca^{2+} oscillations in the mitochondria regulate metabolic enzymes (8). Numerous cytosolic and membrane-bound Ca^{2+}-activated enzymes may be preferentially controlled by local Ca^{2+} signals (9). Colocalization of VSCC with other ion channels leads to the regulation of membrane voltage and cellular excitability (10). Abbreviations: E, Ca^{2+}-activated enzymes; N, nucleus; M, mitochondria; ER, endoplasmic reticulum; G, Golgi apparatus; CSG, secretory granules of the constitutive pathway(s); SG, secretory granules of the regulated pathway(s); EC, endosomal compartments; VDCC, voltage-dependent Ca^{2+} channel; SOC, store-operated Ca^{2+} channel; I_{Cl}, Ca^{2+}-activated chloride channel; I_K, Ca^{2+}-activated potassium channel; PMCA, plasma membrane Ca^{2+}-ATPase; NCX, Na^+/Ca^{2+} exchanger; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; RyR, ryanodine receptor Ca^{2+}-release channel; IP_3R, inositol 1,4,5 trisphosphate Ca^{2+}-release channel; Uni, mitochondrial Ca^{2+} uniporter. Organelles and Ca^{2+} gradients are not drawn to scale.



Ca^{2+} signals in a number of cell types (Hagar et al. 1998; discussed in Section III).

Another characteristic property of the IP_3R family is spontaneous activity (Pozzan et al. 1994). This corresponds to the spontaneous Ca^{2+} release from IP_3-sensitive Ca^{2+} stores (Missiaen et al. 1991) which, together with a passive leak, can be measured when SERCA inhibitors are applied to cells. The activity of these channels can be stimulated through phosphorylation by PKA (Islam et al. 1998; Bird et al. 1993; Liu et al. 1996), and to a lesser extent PKC, CaM, and members of the Src family of non-receptor tyrosine kinases (Jayaraman et al. 1996). It is currently unclear what role these phosphorylation events play in spontaneous- or agonist-generated Ca^{2+} signals from IP_3-sensitive Ca^{2+} stores.

Ryanodine receptor and cADP-ribose Ca^{2+} signalling

Intracellular Ca^{2+} stores containing a receptor/channel sensitive to ryanodine (RyR) were originally thought to reside exclusively in muscle cells. However, the presence of the RyR and its involvement in Ca^{2+} signalling is now appreciated for many non-muscle tissues, and represents a major IP_3-insensitive Ca^{2+} store found in many cell types (Pizzo et al. 1997; Sorrentino and Volpe 1993; Pozzan et al. 1994). Ryanodine-sensitive Ca^{2+} stores are considered to be spatially and functionally distinct from those sensitive to IP_3

(Golovina and Blaustein 1997). Three RyR isoforms are known and, like the isoforms of the IP_3R, may have important functional differences and tissue specificity (Bezprozvanny et al. 1991). Skeletal (RyR-1), cardiac (RyR-2), and brain (RyR-3) isoforms are all found in the CNS (Sorrentino and Volpe 1993), but only RyR-2 and RyR-3 are found in the rat pituitary (Sundaresan et al. 1997). Ryanodine specifically blocks all forms of the RyR at micromolar concentrations, whereas nanomolar ryanodine activates the channel by stabilizing it in a sub-conductance state (Ehrlich et al. 1994). Ryanodine binds to the RyR in its open conformation, making the block use-dependent. This can be important, depending on the basal activity of the RyR-linked stores. Millimolar concentrations of caffeine, which sensitize the RyR to Ca^{2+}, are often used as a probe for its activity.

Like the IP_3R, the RyR is activated by Ca^{2+}. Unlike the IP_3R-1, elevations above resting levels of Ca^{2+} are stimulatory at most physiological concentrations. On this basis of its activation by physiological levels of Ca^{2+}, the RyR was ascribed a primary role in propagating Ca^{2+} waves through the process of Ca^{2+}-induced Ca^{2+}-release (CICR). At this time, it is not clear how CICR terminates, or how Ca^{2+} release from ryanodine-sensitive Ca^{2+} stores can be graded, since one would expect CICR to be prone to regenerative behavior. Several schemes, including adaptation, store depletion, and local inactivation, have been proposed to explain the observed refractoriness of RyR-mediated Ca^{2+} release (Györke and Fill 1993; Sham et al. 1998; Stern 1996). The activity of these channels can also be positively and negatively regulated by phosphorylation (Islam et al. 1998; Wang and Best 1992). Notably, the RyR can be inhibited by CaMK-II (Wang and Best 1992), which could provide a long-term means for modulating the duration of regenerative RyR-mediated Ca^{2+} signals.

The CICR function of ryanodine-sensitive Ca^{2+} stores has been studied in detail. It is now clear that the RyR can also be a primary target for receptor-generated Ca^{2+}-mobilizing chemical messengers (e.g., Tanaka et al. 1998). The endogenous effector for the RyR-2 and RyR-3 isoforms is cyclic adenosine 5′-diphosphate ribose (cADPR; Mészáro et al. 1993). This novel second messenger can be generated by the ubiquitously distributed enzymes, ADP-ribosyl cyclase and CD38 (Galione and White 1994; Pozzan et al. 1994; Kato et al. 1999). cADPR evokes Ca^{2+} signals in a variety of cell types, including pituitary-derived GH_4C_1 cells (Koshiyama et al. 1991). Other second messengers may also target the RyR, including sphingosine-1-phosphate (S1P), 2′-phospho-cADPR and acyl-coenzyme A (Choi et al. 1996; Vu et al. 1996; Fitzsimmons et al. 1997).

cADPR has been proposed as a possible link between nitric oxide and downstream Ca^{2+} release through the RyR (Galione and White 1994). These downstream actions may play a role in several cellular processes including long-term synaptic depression in neurons (Reyes-Harde et al. 1999). Nitric oxide also elicits Ca^{2+} release from ryanodine-sensitive Ca^{2+} stores in pancreatic β-cells (Willmott et al. 1995). These actions may be mediated by cGMP, which is known to participate in Ca^{2+} homeostasis in a number of cell types (Hajnóczky et al. 1994). When viewed together, these reports indicate that RyR-mediated Ca^{2+} release is an important component in several signal transduction pathways. The degree to which RyR and IP_3R Ca^{2+} signals control different cell functions remains to be elucidated.

Nicotinic acid dinucleotide phosphate-sensitive Ca^{2+} stores

Another pyridine nucleotide-derived second messenger, nicotinic acid adenine dinucleotide phosphate (NAADP), has also been studied for its role in releasing Ca^{2+} from intracellular stores. Like cADPR, NAADP is produced by ADP-ribosyl cyclase (Genazzani and Galione 1997). NAADP mobilizes Ca^{2+} from a compartment that is separate from those sensitive to IP_3, cADPR, and thapsigargin, although ATP is still required for its refilling (Genazzani and Galione 1997; Genazzani and Galione 1996). Surprisingly, the putative NAADP-gated intracellular channel is not modulated by $[Ca^{2+}]_c$, indicating that it does not mediate CICR (Chini and Dousa 1996). This finding contrasts with all other intracellular Ca^{2+} channels studied to date, and suggests that the NAADP receptor may play a unique role in shaping Ca^{2+} signals. Recently, a physiological role for the NAADP Ca^{2+} release system in pancreatic acinar cells has been proposed. This report demonstrated that cholecystokinin uses NAADP-sensitive Ca^{2+} stores as the initial trigger for Ca^{2+} signals that are subsequently transduced by IP_3R- and RyR-dependent mechanisms (Cancela et al. 1999). Given the ubiquity of the NAADP-generating enzymes, it is likely that agonist-evoked Ca^{2+} release from NAADP-sensitive store may be common. Interestingly, NAADP-activated Ca^{2+} release from intracellular stores is blocked by diltiazen, nifedipine, BAY K 8644, and verapamil, all classical inhibitors of L-type VDCC (Genazzani Galione 1997). Since, these drugs are well known blockers in many neuroendocrine systems, we may have already, unknowingly obtained evidence of extensive NAADP involvement.

Sphingolipids and metabolites

Sphingolipids are important components of eukaryotic cell membranes that have been implicated in intracellular Ca^{2+} homeostasis and signalling (Zhao et al. 1994; Choi et al. 1996). Certain G-protein-coupled receptors activate sphingosine kinase (Heringdorf et al. 1998), leading to the production of S1P and the subsequent mobilization of Ca^{2+} from IP_3-independent ER stores which may, in some cases, be Ry-sensitive (Choi et al. 1996; Tas and Koschel 1998). Interestingly, S1P can be produced at the ER membrane (Ghosh et al. 1994), suggesting the possibility of localized signalling. The putative ER-derived signals could also mediate communication with other parts of the cell. In agreement with this hypothesis, sphingolipids have been implicated in the regulation of SOCs (Mathes et al. 1998).

S1P is not the only Ca^{2+}-mobilizing sphingolipid. Others have reported IP_3-independent Ca^{2+} mobilization by sphingosyl-phosphocholine (SPC) and not S1P in certain cells (Mao et al. 1996; Yule et al. 1993). These effects may be partially mediated by the RyR-2, as is the case in cardiac muscle (Betto et al. 1997). Notwithstanding, the product of the mammalian SCaMPER (sphingolipid Ca^{2+} release-mediating protein of endoplasmic reticulum) gene encodes a novel protein, unrelated to either the IP_3R or RyR, that mediates SPC-evoked Ca^{2+} release from ER stores (Mao et al. 1996). Taken together, these reports suggest the possibility

that there may be at least two distinct sphingolipid-sensitive intracellular Ca^{2+} stores.

Evidence discussed thus far clearly indicates that multiple functional Ca^{2+} stores can coexist, and suggests that these stores may regulate distinct cellular functions by producing kinetically and spatially unique Ca^{2+} signals. The question of how multiple functional Ca^{2+} stores are segregated within the ER, which is continuous by many accounts, is particularly intriguing (Golovina and Blaustein 1997; Montero et al. 1997). However, it is conceivable that standing gradients in lumenal Ca^{2+}, similar to those already appreciated in the cytosol, may exist. Moreover, the structure of the ER is dynamic and actively regulated by Ca^{2+} (Subramanian and Meyer 1997).

It must be noted that the participation of non-ER Ca^{2+} stores in many of the novel Ca^{2+} signalling systems described above cannot be excluded. Most often these possibilities have not been examined. Determining the anatomical identity of the Ca^{2+} stores mobilized by the second messenger systems noted above is not only expected to yield information regarding their physiological role, but also will undoubtedly be a fruitful and surprising area of inquiry in the future. In addition to sub-compartments of the ER, several candidate Ca^{2+}-regulating organelles, such as the Golgi, secretory granules, nucleus, and mitochondria can be considered. These novel Ca^{2+}-storing organelles are the subject of the following sub-sections.

Golgi-mediated Ca^{2+} signalling

Like the ER, the Golgi stores large amounts of cellular Ca^{2+}, and is an important regulator of Ca^{2+} homeostasis in many cell types, from yeast to mammalian cells (Pezzati et al. 1997; Miseta et al. 1999; Pozzan et al. 1994). Ca^{2+} uptake into the Golgi may occur via thapsigargin-sensitive and -insensitive mechanisms (Pinton et al. 1998; Taylor et al. 1997). The primary candidate for this thapsigargin-insensitive Ca^{2+}-ATPase is the homologue of the yeast Golgi P-type ATPase Pmr1 (Gunteski-Hamblin et al. 1992; Sorin et al. 1997; Pinton et al. 1998). Interestingly, mutants of *Saccharomyces cerevisiae* deficient in Pmr1 have impaired secretory function, suggesting a conserved role for Ca^{2+} homeostasis in the processing of secreted proteins (Rudolph et al. 1989). Experiments using HeLa cells expressing specifically targeted aequorin have shown that the Golgi contains an agonist-sensitive Ca^{2+} store that mediates Ca^{2+} release through the IP_3R-1 (Pinton et al. 1998; Lin et al. 1999). However, the existence of novel Ca^{2+}-sequestering mechanisms, and the expression of Ca^{2+}-binding proteins that are found predominantly or exclusively in the lumen of the Golgi, suggest that the functional properties of this Ca^{2+} store are unique (Lin et al. 1999; Lin et al. 1998; Scherer et al. 1996). Localized Ca^{2+} signals could play a dramatic role in Golgi function, without significantly altering $[Ca^{2+}]_c$. Taken together, these observations demonstrate that the Golgi may be a physiologically important Ca^{2+} store that may generate agonist- or function-specific Ca^{2+} signals. Since the Golgi contains IP_3- and thapsigargin-sensitive components, it is probable that some previous investigations have mistakenly attributed Golgi-evoked Ca^{2+} signals to the ER.

Ca^{2+} signalling in and around the secretory granules

Several lines of evidence suggest that secretory granules, which contain high concentrations of Ca^{2+}, may be the ultrastructural correlates of at least two novel classes of intracellular Ca^{2+} stores (Pezzati et al. 1997; Pozzan et al. 1994). First, many cell types, including pituitary cells, contain a slowly exchanging Ca^{2+} store that can be released by treatments that collapse pH gradients of acidic organelles, but is insensitive to ionomycin and a number of IP_3-generating hormones (Martinez et al. 1996; Shorte et al. 1991; Pozzan et al. 1994). However, this type of secretory granule Ca^{2+} store has not been studied extensively enough to exclude its participation in agonist or antagonist regulation in all neuroendocrine systems.

There is increasing evidence that a second type of secretory granule can be an IP_3- or agonist-sensitive Ca^{2+} store in many cell types, including endocrine (β and δ) and exocrine cells of the pancreas, tracheal goblet cells and *Aplysia* neurosecretory cells (Blondel et al. 1994; Gerasimenko et al. 1996*a*; Nguyen et al. 1998; Jonas et al. 1997). Nguyen and coworkers (1998) recently demonstrated that IP_3 generated oscillations in a subset of secretory granules from rabbit trachea that preceded, and were shown to drive, cytosolic Ca^{2+} fluctuations. Taken together, these reports suggest that the secretory granules may be more important than previously appreciated in the transduction of certain stimuli in some cell types. As is the case with the Golgi Ca^{2+} stores, many past studies might have incorrectly attributed secretory granule Ca^{2+} release to the ER.

Ca^{2+} signalling in the nucleus

Many Ca^{2+}-dependent cellular functions are compartmentalized within the nucleus. Although the understanding of nuclear Ca^{2+} signalling is clearly of great importance, it is poorly understood at present. Specifically, whether the nucleus represents a discrete Ca^{2+} store, or is functionally continuous with the cytosol, is still a contentious issue. Although is has been assumed that the nuclear pore complex would freely pass small molecules, there are now many reports of differential regulation of nuclear and cytoplasmic Ca^{2+} using a variety of cell types and measurement strategies (reviewed in Rogue and Malviya 1999; Korkotian and Segal 1996; Badminton et al. 1996; Al-Mohanna et al. 1994; J.D. Johnson and J.P. Chang, unpublished). The nucleus can generate IP_3 locally. It also possesses the components required to regulate Ca^{2+} independently from the cytosol, including IP_3R, RyR, and SERCA (Gerasimenko et al. 1996*b*). Still, some work has shown that nuclear Ca^{2+} is not maintained differentially from $[Ca^{2+}]_c$ (Rogue and Malviya 1999). The discrepancies found in the literature may be due to cell type differences. A more likely explanation is that the degree to which nuclear $[Ca^{2+}]$ is coupled to, or insulated from, cytosolic Ca^{2+} signals is dynamic and under complex regulatory control. The finding that Ca^{2+} is involved in the normal functioning and restructuring of the nuclear envelope (Stehno-Bittel et al. 1995; Baitinger et al. 1990; Subramanian and Meyer 1997) supports such a hypothesis. Other aspects of nuclear Ca^{2+} signalling that also require clarification included the nature of nucleoplasmic Ca^{2+} homeostasis, the degree of continuity with ER Ca^{2+} stores, the role of the differential distribution of Ca^{2+} signalling pro-

teins on the inner and outer nuclear membranes, the role of the nuclear pore complex, and the interactions between these components. The answers to these questions are bound to have important implications in the regulation of cell function.

Involvement of mitochondria in Ca^{2+} signalling

The capability of mitochondria to transiently sequester large quantities of Ca^{2+} has been appreciated for some time. It is now clear that mitochondria play a very important physiological role in the basal Ca^{2+} homeostasis and agonist-evoked Ca^{2+} signals (Babcock et al. 1997; Pozzan et al. 1994). Resting free intramitochondrial Ca^{2+} levels are only slightly greater than in the cytosol (Duchen 1999). Ca^{2+} is sequestered, at the expense of generating ATP, by energized mitochondria through a uniporter that exploits the electrochemical proton gradient, and is rapidly lost through Na^+/Ca^{2+} exchange and other mechanisms (Duchen 1999; Pozzan et al. 1994; Ichas et al. 1997). The Ca^{2+} uniporter of the mitochondria inner membrane is a high-capacity/low-affinity transport system, and therefore acts as a high-pass filter, shaping the peak and decay phases of transient Ca^{2+} signals that reach micromolar amplitudes (reviewed in Duchen 1999). This Ca^{2+} buffering activity of mitochondria has a profound effect on the spatial and temporal characteristics of Ca^{2+} signals in many cell types, including endocrine cells (Herrington et al. 1996; Hehl et al. 1996). In addition to modulating Ca^{2+} signals generated by extracellular Ca^{2+} influx through VDCC or SOC (Hoth et al. 1997), mitochondria have a dynamic and reciprocal interaction with ER Ca^{2+} release channels (Rizzuto et al. 1993; Rizzuto et al. 1998). Mitochondria can exert dual control on Ca^{2+} signals generated by intracellular Ca^{2+} stores. On one hand, Ca^{2+}-uptake may stabilize steady-state $[Ca^{2+}]_c$ by attenuating accidental Ca^{2+} signals evoked by spontaneous activity of the IP_3R. Conversely, mitochondria can enhance IP_3R-mediated Ca^{2+} signalling by preventing Ca^{2+}-dependent autoinhibition through the sequestration of localized high amplitude Ca^{2+} events (Duchen 1999). ER-mitochondria complexes have been implicated in the propagation of Ca^{2+} waves (Simpson et al. 1997; Csordás et al. 1999). Taken together, these findings indicate that the mitochondria play a primary role in regulating Ca^{2+} signalling.

Mitochondria, together with the other Ca^{2+}-storing organelles that we have discussed, form a complex network for Ca^{2+} signalling. It is their morphological and functional diversity that allow cells to generate sufficiently complex Ca^{2+} signals in response to extracellular stimuli. Examples of the spatial and temporal complexity of Ca^{2+} signals and how information encoded in these patterns may independently regulate specific cellular functions are discussed in the next section.

Section III: Spatial and temporal characteristics of function- and agonist-specific Ca^{2+} signalling

Mechanisms of spatially restricted Ca^{2+} signals

Up to this point, we have reviewed evidence that many functionally distinct Ca^{2+} stores can co-exist within single cells. Much of the value of multiple Ca^{2+} stores would be lost, however, if the Ca^{2+} signals could never remain independent in space. One of the most important determinants of Ca^{2+} signal formation is the properties of the cytosol as a Ca^{2+} buffering medium (discussed above; Jafri and Keizer 1995). Ca^{2+} signals are constrained spatially by the very low effective diffusion rate for the ion in cytosol, when compared to IP_3, for example. Millimolar concentrations of immobile, and to a lesser extent mobile, Ca^{2+} buffering proteins bind Ca^{2+}, and slow the diffusion rate of Ca^{2+} ions by more than one order of magnitude (Allbritton et al. 1992; Tse et al. 1994). Endogenous calcium-binding proteins have low affinity and fast on-rates; Ca^{2+} binding to endogenous buffers reaches equilibrium in less than 1 ms (Roberts 1994; Naraghi et al. 1998; Jafri and Keizer 1995). Ca^{2+} diffusion coefficients have been calculated for a number of cell types, and the estimates are generally in agreement with one another. For example, Ca^{2+} diffuses at a rate of 10 $\mu m^2/s$ in chromaffin cells (Zhou and Neher 1993). However, this diffusion rate is dynamic with respect to ambient $[Ca^{2+}]$. In agreement, Allbritton and coworkers (1992) found that Ca^{2+} diffusion increases from 13 $\mu m^2/s$ to 65 $\mu m^2/s$ when $[Ca^{2+}]_c$ is changed from 90 nM to 1000 nM. This value is only a measure of the buffering capacity of the cytosolic Ca^{2+}-binding proteins, since it was obtained in *Xenopus laevis* oocyte cytosol without contribution of Ca^{2+} sequestering organelles, such as the mitochondria and ER. The effect of these components on Ca^{2+} diffusion is also dynamic, and depends on the local $[Ca^{2+}]_c$ (Duchen 1999; Pozzan 1994). The physical presence of organelles can also have a large effect on the apparent Ca^{2+} diffusion rate (Naraghi et al. 1998). These properties of the cytosol have important consequences for the generation of Ca^{2+} signals.

Just as individual neurons in the brain must be able to fire independently, as well as in concert, the ability of cells to independently regulate Ca^{2+} in spatially distinct regions is a prerequisite for spatial coding of Ca^{2+} signals (discussed below). Can cells generate persistent, as well as transient, localized Ca^{2+} fluctuations in the continuous cytosolic space? Ca^{2+} gradients can arise when the rate of Ca^{2+} influx into a space exceeds the rate of diffusion out of that space (Machaca and Hartzell 1999). Since the diffusion of Ca^{2+} is comparably slow, steep local gradients may be common.

Transient Ca^{2+} gradients of up to 100 μM do occur around the openings of Ca^{2+} channels or clusters of Ca^{2+} channels in both excitable and non-excitable cells. Ca^{2+} signals result from the coordinated recruitment of these elementary events, which are referred to as Ca^{2+} sparks, or Ca^{2+} puffs (Berridge 1997). Since the IP_3R and RyR are activated by Ca^{2+}, individual Ca^{2+}-release channels are engaged into the Ca^{2+} signal by the local diffusion of Ca^{2+} (Berridge 1997). This process is facilitated by their respective second messengers. In many cell types, these events are initiated in trigger zones, which often have specific isoforms of the IP_3R or higher resting Ca^{2+} (Ito et al. 1997). For example, the specific properties of the IP_3R-3 isoform are favorable for initiating Ca^{2+} waves in the trigger zone of pancreatic acinar cells (Nathanson et al. 1994; Thorn et al. 1993). The high resting Ca^{2+} in these regions also increases the Ca^{2+} signal gain in response to agonists. Once a Ca^{2+} signal is initiated, it can be regenerative in both time and space, much like action potential (Berridge 1998). Unlike an action potential, there is evidence that Ca^{2+} signals can be graded.

The propagation of Ca^{2+} signals often takes the form of a non-decremental wave. Two types of Ca^{2+} wavefronts, saltatory and continuous, have been recorded from a variety of cell types (Dawson et al. 1999). Rather than arising via different mechanisms, recent modeling work suggests that both types may originate through a common mechanism. An interesting prediction of the model proposed by Dawson and colleagues (1999) is that the propagation of Ca^{2+} waves is continuous if the release of Ca^{2+} from stores is rate-limiting, but saltatory if the diffusion from one release site to the next is rate-limiting. Given that the IP_3R and RyR (and their isoforms) have different unitary conductance, their relative distribution may dictate the type of Ca^{2+} signals generated by a specific cell or region of a cell.

As one might expect, the Ca^{2+} signalling machinery of the cell is distributed in a strategic manner within cells. In addition to the spatial heterogeneity of Ca^{2+}-storing organelles (Simpson et al. 1997), all types of Ca^{2+} channels and pumps on the plasma membrane or membranes of organelles have been shown to be spatially localized (Lee et al. 1997; Kasai et al. 1993). IP_3R and RyR are organized in clusters in a variety of cell types (Wilson et al. 1998; Mak and Foskett 1997). Recent evidence suggests that this phenomenon can be acutely regulated by Ca^{2+} in rat basophilic leukemia cells (Wilson et al. 1998) suggesting that the mechanisms by which cells generate Ca^{2+} signals can be modulated by extracellular signals. The redistribution of Ca^{2+} signalling components could provide a general mechanism for regulating the excitability of neuroendocrine systems. In addition to regulating excitability of the intracellular Ca^{2+} signalling network, modifications to the routes by which Ca^{2+} waves travel could conceivably allow cells to switch the cellular functions that are regulated by a given stimulus. How cells control signal specificity by compartmentalizing function is the subject of the next sub-section.

Spatial compartmentalization of Ca^{2+}-dependent cellular functions

One method to ensure that localized Ca^{2+} signals regulate cellular functions with specificity is to spatially segregate the cellular functions. Spatial coding of Ca^{2+} signals is well established in neuronal models because of the obvious compartmentalization of cellular functions in the dendrites, soma, axon, and synapse. There is ample evidence that this strategy is common to all cell types. Many Ca^{2+}-activated enzymes are compartmentalized or associated with specific membranes. Importantly, the spatial distribution of activated Ca^{2+} effector proteins, such as CaM and CaMK-II, reflect the spatial pattern of Ca^{2+} signals (Inagaki et al. 1997; Craske et al. 1999). This suggests that cells are capable of decoding spatial information.

Key regulators of cell excitability such as the Ca^{2+}-activated K^+ channel (Marrion and Tavalin 1998) are known to exist in close proximity to Ca^{2+} channels, forming isolated signalling complexes. Steep subplasmalemmal Ca^{2+} gradients, which are only detected with membrane targeted Ca^{2+} indicators, are correlated with the activity of Ca^{2+}-activated Cl^- channels in *Xenopus* oocytes (Machaca and Hartzell 1999). It was recently demonstrated that Ca^{2+} influx through SOC specifically regulates closely associated Ca^{2+}-inhibitable adenylyl cyclase (Fagan et al. 1998). Interestingly, hormone receptors and adenylate cyclases have been found compartmentalized in subplasmalemmal structures (Schwencke et al. 1999).

Ca^{2+} signals can also be targeted to specific organelles without affecting the bulk cytosol. In many cell types, the mitochondria respond to localized, high-amplitude Ca^{2+} signals, delivered by closely positioned ER Ca^{2+} release channels, that are not observed in gross measurements of $[Ca^{2+}]_c$ (Rizzuto et al. 1998; Rizzuto et al. 1993). Ca^{2+} signals transmitted through the mitochondria are responsible for metabolic pacing (Hajnóczky et al. 1995; Duchen 1999) and steroid synthesis (Capponi et al. 1988). Spatial Ca^{2+} signalling may also be important in the differential regulation of gene expression. Ca^{2+} acts through at least two different signal transduction pathways to regulate hippocampal gene expression. Apparently, a CaM mediated pathway is only required when Ca^{2+} influx occurs through VDCC, but not ionotropic *N*-Methyl-D-aspartate receptors (Bading et al. 1993).

Spatial coding can also solve the problems associated with the independent regulation of the different sites on the extended secretory pathway, outlined in Section I. The differential effects on gene expression, protein synthesis, and protein processing can be reconciled by the functional compartmentalization of these functions in the nucleus, ER, and Golgi. However, the solution to how the regulated and constitutive secretory pathways may be differentially controlled is less obvious. Some data supports the possibility that the regulated and constitutive secretory pathways may be segregated, in addition to having possible differences in their sensitivity to Ca^{2+}. Evidence for this comes from studies in neuroendocrine AtT-20 cells and from the constitutive and regulated secretion of von Willebrand factor from human endothelial cells (Rivas and Moore 1989; Sporn et al. 1989). By extrapolation, these findings indicate that localized Ca^{2+} signals can trigger the fusion of constitutive granules, without affecting the fusion of other cellular membranes.

There is growing evidence that spatially restricted exocytosis in neuronal active zones and polarized secretory cells are not exceptional cases and that regulated exocytosis can be spatially restricted in many cell types (Carabelli et al. 1998; Schroeder et al. 1994; Sporn et al. 1989). Colocalization of catecholamine secretion with subplasmalemmal Ca^{2+} domains has been directly observed in chromaffin cells (Robinson et al. 1995). On the other hand, a recent study using GFP-tagged clathrin demonstrated that spatially restricted endocytosis also occurs (Gaidarov et al. 1999). Taken together, these results suggest that many types of cells may compartmentalize Ca^{2+}-dependent secretion and, separately, membrane retrieval to avoid unwanted cross-talk with other Ca^{2+}-dependent cellular functions.

The multiplicity of Ca^{2+} action suggests that inhibitory neuroendocrine regulators of hormone synthesis or release may also act at many potential target sites. For example, the lumens of Ca^{2+}-storing organelles may represent a potential site of neuroendocrine regulation of agonist-stimulation. Specifically, modulation of the lumenal Ca^{2+} environment in secretory granules would have marked effects on the secretion of hormones at both pre- and post-fusion stages (see Section I). Moreover, biochemically distinct populations of

secretory granules could be targeted. Several lines of evidence suggest that many neuroendocrine inhibitors act at important late sites in the secretory pathway. For instance, the Ca^{2+}-dependent phosphatase, calcineurin, mediates the inhibition of insulin secretion by somatostatin, galanin, and adrenaline, through a mechanism that is distal to the depolarization-evoked Ca^{2+} signal (Renström et al. 1996). It is interesting to note that Ca^{2+}-dependent enzymes can both stimulate and inhibit secretion. Perhaps the unique Ca^{2+} signal leading to the activation of calcineurin is insufficient to activate Ca^{2+}-dependent pro-exocytosis pathways. Further work is necessary before the complexity of Ca^{2+} involvement, both positive and negative, in the extended secretory pathway can be understood.

It is also interesting to consider the possibility of spatial heterogeneity of Ca^{2+} signals traveling within the lumens of organelles. The problems posed by the continuity of the lumen of the ER are analogous to those faced by the cytosol with respect to the creation of spatially localized Ca^{2+} signals. It is becoming clear that the cytosol uses the strategic and dynamic placement of Ca^{2+} pumps and channels to generate Ca^{2+} fluctuations with significant spatial and temporal complexity (Wilson et al. 1998; Lee et al. 1997; Kasai et al. 1993). This process is coupled to a very low diffusion rate for Ca^{2+}, and possibly, the non-uniform distribution of buffering capacity in the cytosol. The lumen of the ER, which is heterogeneous in many respects, may use a similar strategy to transduce complex Ca^{2+} signals and modulate discrete lumenal targets. The spatial and temporal characteristics of lumenal Ca^{2+} signals may, in turn, have important roles in modulating cytosolic Ca^{2+} signals in a variety of cell types, as predicted by several theoretical and experimental studies (Jafri and Keizer 1995; Lukyanenko et al. 1999; Nguyen et al. 1998). The possibility that complex Ca^{2+} signals in the lumen of Ca^{2+} stores may regulate cytosolic Ca^{2+} signalling in a reciprocal manner, requires further attention.

Further research will be required to resolve the molecular mechanism by which Ca^{2+} signals and Ca^{2+}-dependent cellular functions are colocalized, in the cytosol and in organelle lumens. There is a potential role for scaffold complexes, like those that regulate interactions of proteins in the MAPK pathways (Garrington and Johnson 1999), in promoting the physical association of Ca^{2+}-binding and -signalling proteins with their targets. The cytoskeleton may also be prominently involved in the spatial aspects of Ca^{2+} signalling. There is evidence that the cytoskeleton mediates the coordination of Ca^{2+} mobilization between multiple Ca^{2+} stores (Hajnóczky et al. 1994) and the generation of agonist-evoked Ca^{2+} signals (Ribeiro et al. 1997). It is clear that the spatial complexity of Ca^{2+} signals is necessary to support the independent regulation of Ca^{2+}-dependent cell functions. Equally complex are the temporal waveforms of agonist-generated Ca^{2+} signals, which are considered next.

Temporal coding of Ca^{2+} signals: Duration and amplitude

One important characteristic of physiological, high-amplitude Ca^{2+} signals, is that they must be transient to avoid Ca^{2+} cytotoxicity. Many factors shape the waveform of Ca^{2+} transients. The duration of Ca^{2+} signals may be controlled by specific characteristics of the IP_3R and RyR isoform(s) involved (detailed in Section II), and the rate of Ca^{2+} removal from the cytosol. In instances when prolonged signals are required, cells generally exhibit biphasic Ca^{2+} signals or elevations of Ca^{2+} with suitably low amplitude. The activation of protein kinases, such as CaMK, also ensures that the influence exerted by extracellular signals on intracellular processes persists (MacNicol et al. 1990). Notwithstanding, there are several notable examples where cell functions are controlled by the duration of Ca^{2+} signals per se. In T lymphocytes, NF-κB and Jun amino-terminal kinase are specifically stimulated by transient Ca^{2+} signals with high amplitude, while NFAT is activated by low-amplitude, long-duration Ca^{2+} signals (Dolmetsch et al. 1997). Recently, the translocation of PLA_2 from the cytosol to the nuclear envelope/ER and its subsequent activation were shown to be insensitive to brief Ca^{2+} transients, but is dependent on Ca^{2+} signals with a duration above 1 min (Hirabayashi et al. 1999). Taken together, these reports suggest that the duration of Ca^{2+} signals can encode specific information.

The most obvious mechanism for regulating specific functions is by modulating the amplitude of Ca^{2+} signals. The range of affinities for Ca^{2+} shown by the suite of effector proteins found in cells extends over several orders of magnitude. This suggests that cells can selectively transduce information by targeting specific effector proteins using the amplitude of Ca^{2+} signals. Many examples of this are found in the literature. Micromolar Ca^{2+} signals were required to activate exocytosis in pancreatic acinar cells. In contrast, some membrane ion channels were specifically activated by lower amplitude Ca^{2+} transients, suggesting that the amplitude of the Ca^{2+} signal can encode specificity (Ito et al. 1997).

In addition, the amplitude of the steady-state Ca^{2+} levels can have profound effects on other signal transduction pathways. For example, whether the Na^+-K^+-ATPase is stimulated or inhibited by PKC or PKA depends on the local ambient $[Ca^{2+}]_c$ (Cheng et al. 1999). The resting Ca^{2+} concentration plays an important role in the generation of specific Ca^{2+} oscillations by agonists in pancreatic acinar cells (Toescu et al. 1993). Adjusting $[Ca^{2+}]_c$ slightly upwards increases the probability that CICR will occur. This effectively sets the cytoplasm closer to the threshold for Ca^{2+} signalling activity. Modulating the gain of the Ca^{2+} signalling system may a common mechanism regulating cellular excitability.

Temporal coding of Ca^{2+} signals: Oscillation frequency

Ca^{2+} oscillations are one of the most common Ca^{2+} signal waveforms seen in agonist-stimulated cells (Petersen et al. 1994). The ER is an excitable membrane capable of generating Ca^{2+} signals that are regenerative in both space and time (reviewed in Berridge 1998). The mechanisms of Ca^{2+} oscillations have received much experimental and theoretical attention. The interested reader is referred to several enlightening reviews (Fewtrell 1993; Sneyd et al. 1995; Petersen et al. 1994). Some of these models incorporate Ca^{2+} stores as a single entity. However, since the evidence summarized above suggests that most cells possess multiple intracellular Ca^{2+} stores, each with distinct properties, the situation in living cells may be considerably more complex. In the rest of this section, we will describe recent advances in the understanding of the functional consequences of Ca^{2+} oscillations.

Ca^{2+} oscillations and their frequency modulation have been found to regulate many cellular functions. For example, in rat gonadotropes, as in many other cell types, each oscillation in $[Ca^{2+}]_c$ is associated with a burst of gonadotropin exocytosis (Tse et al. 1993). Ca^{2+} oscillation frequency is dose-dependent in many cells types (Woods et al. 1986; Petersen et al. 1994; Tse et al. 1993). Perhaps the most powerful evidence of Ca^{2+} signal coding through frequency comes from studies of Ca^{2+}-regulated gene expression. Dolmetsch and colleagues (1998) controlled the frequency of Ca^{2+} transients in T-lymphocytes by rapidly changing extracellular $[Ca^{2+}]$ while SOCs were kept open using thapsigargin. They demonstrated that high frequency oscillations activate the transcription factors, NF-AT, Oct/OAP, and NF-κB, while lower frequency oscillations only activated NF-κB. Similar frequency-dependence of Ca^{2+}-regulated gene expression was also reported for rat basophilic leukemia cells challenged repeatedly with liberated caged IP_3 (Li et al. 1998). Moreover, Ca^{2+}-sensitive mitochondrial dehydrogenases are preferentially activated by oscillations in mitochondria, when compared to sustained elevations (Hajnóczky et al. 1995). Similarly, angiotensin II generates frequency-coded mitochondrial Ca^{2+} oscillations in adrenal glomerulosa cells that may be functionally important in aldosterone synthesis (Pralong et al. 1994).

Ca^{2+} oscillations on a larger time scale are also coded by frequency. For example, developing *Xenopus* neurons exhibit Ca^{2+} waves and spikes, in the growth cones and somata, respectively. The different frequencies of Ca^{2+} oscillations in these compartments lead to specific modulation of neurite outgrowth and differentiation (Gu and Spitzer 1995). Pulsatility on a larger time scale is also common in neuroendocrine systems. For example, the frequency coding of fluctuations in gonadotropin-releasing hormone release from the hypothalamus, controls gonadotropin release and gene expression in the pituitary. As might be expected, pulses of elevated $[Ca^{2+}]_c$ are more effective than a sustained rise in regulating gene expression in gonadotropes (Haisenleder et al. 1997). Interestingly, the optimal pulse frequency was different between the two gonadotropic hormones, luteinizing hormone and follicle-stimulating hormone, suggesting a novel mechanism for their differential neuroendocrine regulation.

How are Ca^{2+} oscillations decoded? Illumination of this very important question has recently come from elegant work of De Konink and Schulman (1998), who recently demonstrated that one of the major Ca^{2+} signal effector proteins, CaM kinase II, has the intrinsic property of being tuned to respond optimally to Ca^{2+} oscillations with higher frequency, in vitro. Others have found that the translocation and accumulation of CaM in the nucleus of pancreatic acinar cells is best stimulated by agonist-generated Ca^{2+} oscillations (Craske et al. 1999). These results demonstrate, unambiguously, that Ca^{2+} effector proteins have the capacity to decode temporally complex Ca^{2+} signals with fidelity.

Temporal coding of Ca^{2+} signals: Rate of rise

Another temporal feature of Ca^{2+} signals that could encode information is their rate of rise. Hernandez-Cruz and colleagues (1997) recently described a phenomenon in neurons where the activation of CICR depends on the rate of rise in the initial Ca^{2+} event. Since CICR represents a common mechanism for propagating Ca^{2+} signals, this finding is likely to have widespread implications. The rate of rise of Ca^{2+} signals has been considered important for the regulation of Ca^{2+} dependent functions, including exocytosis. It has been proposed that the rate of rise of secretagogue-induced Ca^{2+} signals may be an important determinant of prolactin secretion from GH_4C_1 cells (Sato et al. 1990). The rate of rise is dose-dependent in several agonist-stimulated cell types (Johnson et al. 1999). Also, the simultaneous measurement of membrane capacitance and $[Ca^{2+}]_c$ in rat gonadotropes has revealed that fast-rising Ca^{2+} signals are most effective in activating exocytosis (Tse et al. 1997). Using *Xenopus* oocytes, Machaca and Hartzell (1999), compared the effects of rapid IP_3R-mediated and slower SOC-generated Ca^{2+} signals on several classes of Ca^{2+}-activated Cl^- currents. Their data indicated that the rate of rise was a determining factor in the selective activation of the currents. These observations strongly suggest the possibility that information may be coded in the rate of rise of Ca^{2+} signals.

Temporal coding of Ca^{2+} signals: Waveform

Generally, Ca^{2+} signals can be described as biphasic, monophasic, and oscillatory, although many cell types show hybrid profiles. Individual cell types may generate multiple waveforms (Johnson et al. 1999; Stojilkovic et al. 1992). It is commonly thought that the biphasic Ca^{2+} signals, such as those evoked by agonists in many cell types, are related to the sequential activation of distinct signalling pathways. The Ca^{2+} signal waveform elicited by agonists is dose-dependent in many cell types (Johnson et al. 1999; Stojilkovic et al. 1992). The relative occurrence of various Ca^{2+} waveforms is under endocrine and developmental control, implying that the presence of multiple Ca^{2+} signal profiles is physiologically important (Tomić et al. 1994). We have recently observed that higher-amplitude, biphasic Ca^{2+} signals are most common during late sexual maturation in goldfish gonadotropes (J.D. Johnson and J.P. Chang, unpublished observations). Interestingly, a unique morphological subtype of gonadotropes may be responsible for these specialized Ca^{2+} signals. We have postulated that cells can use duration, amplitude, frequency, and rate of rise coding of Ca^{2+} signals. It is unclear, at this time, whether or not cells can receive additional, more complex information from the waveform of the Ca^{2+} signals. Biphasic Ca^{2+} signals, which are common to many neuroendocrine cell types, can be thought of as a transient Ca^{2+} spike, usually from intracellular stores, followed by a sustained plateau of extracellular Ca^{2+} influx (Catt and Stojilkovic 1989). Since these distinct components have different origins and since amplitude and duration can code for specific functions, it is highly likely that their combination can transduce important information. The experimental generation of Ca^{2+} elevations with more complex profiles that mimic physiological agonist-induced Ca^{2+} signals is required before these possibilities can be tested further.

Whether similar machinery is responsible for all the Ca^{2+} signal profiles in a single cell types, is not known. Could the functional diversity of intracellular Ca^{2+} release channels play a role in generating these complex patterns? An answer to this question has recently been proposed by Miyakawa et al. (1999) who used B lymphoma cells that had been geneti-

cally engineered using homologous recombination to express single IP_3R subtypes or specific combinations of IP_3R subtypes. Activation of the B cell receptor evoked an immediate single spike in cells expressing only IP_3R-1 and IP_3R-3, while robust oscillations were seen in the IP_3R-2-expressing cells (Miyakawa et al. 1999). These results indicate that the distinct properties of intracellular Ca^{2+}-release channels can lead to dramatically difference Ca^{2+} signal waveforms in situ.

It is possible that the combination of spatial and temporal coding may also be important. Few studies have investigated this possibility in earnest. However, simultaneous and independent Ca^{2+} signals have been measured in distinct regions of single cells. For example, localized Ca^{2+} oscillations in response to low doses of agonist have been demonstrated in pancreatic acinar cells (Thorn et al. 1993). In another example, activation of purinergic receptors elicited spatially segregated oscillations with independent frequencies in single smooth muscle cells (Mahoney et al. 1993). Although whole-cell oscillations are rare in goldfish gonadotropes, we have observed similar phenomena, where Ca^{2+} signals exhibit distinct temporal profiles simultaneously in separate parts of the cell (J.D. Johnson and J.P. Chang, unpublished observations).

Other potential modes of temporal Ca^{2+} signal coding have not been investigated experimentally. It is conceivable that Ca^{2+} signals could encode information using delay with respect to other cellular events. By analogy to the nervous system, could information be encoded in the spatio-temporal coincidence with other signalling molecules or with other Ca^{2+} signals? Perhaps the relationships between the phases of multiple independent Ca^{2+} oscillations could also encode information. These possibilities illustrate the complexity of signals that can be attained when spatial and temporal Ca^{2+} signal coding are combined. When one considers the number of Ca^{2+}-dependent cellular functions that are known to occur simultaneously, it is apparent that these interactions may be required, if not unavoidable.

Agonist-specific Ca^{2+} stores and Ca^{2+} signatures

Thus far, we have argued that the number of Ca^{2+}-dependent cellular functions necessitates a complex system for the generation of Ca^{2+} signals, and that cells do possess such capabilities. What physiological evidence substantiates this proposal? Many examples of this are beginning to emerge in the literature. For example, in human neutrophils, chemoattractant stimuli evoke Ca^{2+} release from the centrally located, IP_3-sensitive Ca^{2+} stores, whilst peripheral stores, which are IP_3-insensitive, are involved in cell adhesion (Pettit and Fay 1998). The diversity of Ca^{2+} signals has also been examined in several other secretory cell types, including pancreatic β-cells, pancreatic acinar cells, adrenal medullary cells, and pituitary cells, as well as cell lines derived from these tissues. Using selected examples from these model systems, we will attempt to demonstrate the existence of agonist- and function-specific Ca^{2+} signalling, and explore the potential for further studies in these areas.

The pancreatic β-cell is an excellent model to compare agonist-specific Ca^{2+} signatures and to study multiple Ca^{2+}-dependent cellular functions. Multiple Ca^{2+}-mobilizing agonists, with distinct signalling pathways, act on pancreatic β-cells. This permits the direct comparison of Ca^{2+} signals originating from VDCC, IP_3R, and RyR, and their physiological outcomes (Misler et al. 1992; Holz et al. 1999; Willmott et al. 1995; Islam et al. 1998). Although the signal transduction cascades leading to the activation of VDCC and IP_3 have been characterized, the mechanism by which ryanodine-sensitive Ca^{2+} stores are activated has only recently been elucidated. Although some studies have failed to implicate cADPR using pharmacological methodologies (Webb et al. 1996), Kato and co-workers (1999) recently demonstrated that Ca^{2+} signals and insulin secretion are inhibited by ~50% in response to high-glucose stimulation, but not membrane depolarization, in $CD38^{-/-}$ mice. The discrepancy between this and previous reports may be due to the differences in methodologies and (or) species or mouse strains used.

In addition, the involvement in β-cell function of several classes of Ca^{2+}-storing organelles, including the ER, secretory granules, and mitochondria has been investigated (Blondel et al. 1994; Bode et al. 1996). Among these stores, the prominent role of mitochondria in metabolism-secretion coupling has attracted considerable attention (Maechler et al. 1997; Duchen 1999). However, the relationship between this Ca^{2+} store, and other Ca^{2+} stores has not been investigated. The β-cell would be an ideal model to examine the functional consequences of quasi-synaptic mitochondrial-ER interactions reported in other cell types (Csordás et al. 1999; Rizzuto et al. 1998). Other than insulin secretion and metabolism, several other Ca^{2+}-dependent functions have been measured in β-cells, including proinsulin processing in the ER (Guest et al. 1997) and apoptosis (Zhou et al. 1998; Efanova et al. 1998). How, or if, Ca^{2+} regulates these cell functions, as well as secretion, independently of each other has not been investigated.

Several novel aspects of Ca^{2+} signalling have also been studied in pancreatic acinar cells. The spatial, temporal, and possibly mechanistic, differences between Ca^{2+} signals elicited by multiple agonists, including acetylcholine and cholecystokinin, and their relation to frequency and amplitude coding, have been explored in detail (Petersen et al. 1994; Ito et al. 1997,1999; Thorn et al. 1993; Lee et al. 1997; Kasai et al. 1993). One highlight of this model system is that the participation of several Ca^{2+}-releasing second messengers have been proposed, including IP_3, cADPR, NAADP, sphingosine phosphate, and acyl-coenzyme A (Yule et al. 1993; Fitzsimmons et al. 1997). Although the novel Ca^{2+}-releasing molecules release Ca^{2+} from an IP_3-insensitive pool, it is not clear whether these agents release Ca^{2+} from overlapping stores. Interestingly, the secretory granules contain Ca^{2+} stores sensitive to IP_3 and cADPR (Gerasimenko et al. 1996). The degree to which these Ca^{2+} signalling pathways interact to control secretion and other cell functions requires additional work.

Work on chromaffin cells of the adrenal medulla has advanced our understanding of how the spatial and temporal complexities of Ca^{2+} signalling may regulate cell function. The Ca^{2+}-requirement of several facets of the exocytotic pathway, as well as Ca^{2+}-dependent endocytosis, has been investigated (Neher and Zucker 1993). Nicotinic activation of L-type VDCCs, in chromaffin-derived PC12 cells, stimulated both chromogranin A transcription and catecholamine

secretion, while Ca^{2+} release from intracellular stores activated exocytosis, but not gene transcription (Cheek et al. 1989; Tang et al. 1997), suggesting that Ca^{2+}-dependent cell functions are controlled independently by Ca^{2+} signals with distinct origins. In addition to an important role for VDCC in this cell type, several agonists, including muscarinic, histamine, bradykinin, angiotensin II, and pituitary adenylate cyclase-activating polypeptide (PACAP) release Ca^{2+} from intracellular sources (Cheek and Barry 1993). The ER of chromaffin cells contains both IP_3R- and RyR-dependent Ca^{2+} signalling pathways (Alonso et al. 1999; Tanaka et al. 1998). For example, PACAP is a secretagogue that mobilizes Ca^{2+} from RyR, but not IP_3,-sensitive Ca^{2+} stores (Tanaka et al. 1998), although longer-term stimulation of catecholamine secretion requires influx of extracellular Ca^{2+} (Taupnot et al. 1998). Taken together, these results suggest that the mechanism of Ca^{2+} signalling has important functional consequences. Additional work will be needed to determine if the unique Ca^{2+} signals evoked by certain agonists can regulate specific cell functions in this cell type.

The discovery of colocalized secretion and Ca^{2+} signalling domains suggests that spatial Ca^{2+} signalling may be important in chromaffin cells (Robinson et al. 1995). Nicotinic activation of VDCC induces global Ca^{2+} rises, emanating from the plasma membrane, that evoke exocytosis. In contrast, non-secretagogue stimulation with muscarinic agonist activates IP_3-R mediated Ca^{2+} signals of a similar total cellular amplitude, but that do not propagate from the location at which they are initiated (Cheek et al. 1989). This result cannot be accounted for by proposing that only VDCC activation can stimulate secretion, since exocytosis can occur in voltage-clamped cells (Augustine and Neher 1992; Robinson et al. 1996). Instead, it is possible that the muscarinic Ca^{2+} signal is segregated from either a trigger zone that could evoke the global Ca^{2+} signal through CICR, or a compartmentalized exocytosis-specific region (D'Andrea and Grohovaz 1995). SOC and exocytotic sites may also be colocalized. Despite generating only a modest elevation in $[Ca^{2+}]_c$, thapsigargin-induced store depletion triggers significant secretion through the activation of SOC (Cheek and Thastrup 1989; Fomina and Nowycky 1999). When assembled, these findings indicate that the chromaffin cell will be a fruitful model in the study of Ca^{2+}-dependent regulation of multiple cellular functions, and in the mechanism of multiple unique agonist Ca^{2+} signatures.

Function- and agonist-specific Ca^{2+} signalling is also being investigated in mammalian and non-mammalian pituitary cells, which are under multifactorial regulation. One class of pituitary cells where the specificity of Ca^{2+} signalling has been examined is the gonadotropes, which synthesize and secrete two gonadotropic hormones, GTH-I and GTH-II (also known as luteinizing hormone and follicle-stimulating hormone). As discussed above, frequency-coded Ca^{2+} signals have been implicated in the secretion of GTH-II and differential control of GTH-I and GTH-II synthesis (Tse et al. 1993; Haisenleder et al. 1997). It seems likely that the spatial and temporal characteristics of agonist-evoked Ca^{2+} signals may also be responsible for the differential control of GTH-I and GTH-II secretion. Gonadotropes have also been a model for comparing agonist-specific Ca^{2+} signals (Stojilkovic and Catt 1992).

In our laboratory, we have recently compared, in some spatial and temporal detail, the different Ca^{2+} signals generated by two closely related neuropeptide agonists of the goldfish gonadotrope, the gonadotropin-releasing hormones (sGnRH and cGnRH-II; Johnson et al. 1999; reviewed Chang et al. this issue). Previously we have found that sGnRH and cGnRH-II act through partially distinct signalling pathways in this cell type. The prolonged Ca^{2+} signals which are more commonly generated by sGnRH, when compared to cGnRH-II, correlate with differences in both the bioactivity and signalling cascades between these two neuropeptides (Johnson et al. 1999). In goldfish gonadotropes, only sGnRH activates PLA_2, an enzyme that has been shown to be dependent on the duration of Ca^{2+} signals on other cell types (Hirabayashi et al. 1999). Likewise, sGnRH shows preferential activation of the transcription of gonadotropin subunits (Khakoo et al. 1994); gene expression can also be coded by Ca^{2+} signal duration in some cell types (Dolmetsch et al. 1997). Alternatively, it is possible that the sGnRH-specific Ca^{2+} signal profiles may be due to Ca^{2+} mobilization regulated by AA (Rzigalinski et al. 1996; Shuttleworth 1996; Graber et al. 1997). In any case, it is clear that the unique features of agonist-specific Ca^{2+} signals play an important role in information transduction within cells.

Conclusions

All cells use similar Ca^{2+}-signalling machinery composed of conserved buffers, pumps, and channels. The complexity and cell specificity is manifested in the details of how these components are arranged and activated in the generation and modulation of Ca^{2+} signals. Ca^{2+} signalling is complex, and at the same time, ingenious in its simplicity. Does Ca^{2+} signalling confer specific advantages when compared to information transduction using more structurally complicated signalling molecules? If so, is this the reason for its involvement in countless cellular functions? Although these questions cannot presently be answered, considerable progress has recently be made with regard to how cells accomplish agonist- and function-specific Ca^{2+} signalling.

In this article, we have suggested that the existence of multiple spatially and functional distinct Ca^{2+} stores helps cells solve the potential problems associated with the simultaneous control of many targets. Clearly, the diverse properties of Ca^{2+} stores that can act independently, or together, provide the necessary functional richness to create complex Ca^{2+} signals. These Ca^{2+} signals may be unique to a particular agonist or targeted to a single cellular function, or both. However, evidence that parallel, but independent, Ca^{2+} signals can simultaneously modulate various cellular functions in single intact cells remains elusive.

To test this hypothesis, we will require investigations that measure multiple Ca^{2+}-dependent cellular functions, and compare the details of Ca^{2+} signals in response to multiple agonists, with high spatial and temporal resolution. Although they have not been used in combination, the tools exist to measure exocytosis, secretory pathway function, gene expression, cellular metabolism and the translocation of Ca^{2+} effector proteins with high temporal resolution. In addition, there is a requirement for methods that manipulate $[Ca^{2+}]$ in a more delicate and spatially restrictive manner

than is currently possible with flash photolysis of caged Ca^{2+}. Another important avenue of investigation will be to use computer assisted modeling. Due to the complexity of multiple, simultaneously interacting Ca^{2+} signalling cascades, mathematical modeling approaches may be very advantageous in assembling these findings and generating testable hypotheses. But this is only the beginning of a bigger story. What part does this complexity at the cellular level play in the functioning of whole organisms?

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