Perinuclear Ca2+ regulation of nuclear Ca2+ ion transport

BY

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A Thesis

Submitted to the Faculty of Graduate Studies
In Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

Department of Oral Biology
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Above all, Dr. Gilchrist, no call me James ... ;D , thanks for giving me the opportunity to learn, grow, and find my strengths and weaknesses. For that I will always be in your debt. As you have always said, "This is a maturation process". Through the good and the bad I've learned something about myself, and that is all I have ever wanted. It was a crazy ride and I would not trade it for anything.

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Laboratory members, past and present, you have all given me an opportunity to see a reflection of myself, in the way I have dealt with all of you, and I have learned some very valuable lessons about myself, a heart-felt thanks. For the friendship, and the "big brother" technician, I was lucky to have had Tom around the lab. Chris, even though you weren't here at the end, your impact during our "haze-filled" time is immeasurable. Penny and Mark, I am also thankful for both of your friendship and camaraderie. The various members of the Pierce Lab (you all know who you are), thanks for letting me be a constant parasite on your lab resources, it has always been fun.

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Abstract

Regulation of nucleoplasmic calcium (Ca^{2+}) concentration may occur by the mobilization of perinuclear luminal Ca^{2+} pools involving specific Ca^{2+} pumps and channels in both inner and outer perinuclear membranes. To determine the role of perinuclear luminal Ca^{2+}, we examined freshly cultured 10 day-old embryonic chick ventricular cardiomyocytes. We obtained evidence suggesting the existence of the molecular machinery required for the bi-directional Ca^{2+} fluxes using confocal imaging techniques. Embryonic cardiomyocytes were probed with antibodies specific for ryanodine-sensitive Ca^{2+} channels (RyR2), sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA2) -pumps, and fluorescent BODIPY derivatives of ryanodine and thapsigargin. Using immunocytochemistry techniques, confocal imaging showed the presence of RyR2 Ca^{2+} channels and SERCA2-pumps highly localized to regions surrounding the nucleus, referable to the nuclear envelope. Results obtained from Fluo-3, AM loaded ionomycin-perforated embryonic cardiomyocytes demonstrated that gradual increases of extranuclear Ca^{2+} from 100 to 1600 nM Ca^{2+} was localized to the nucleus. SERCA2-pump inhibitors thapsigargin and cyclopiazonic acid showed a concentration-dependent inhibition of nuclear Ca^{2+} loading. Furthermore, ryanodine demonstrated a biphasic concentration-dependence upon active nuclear Ca^{2+} loading. The concomitant addition of thapsigargin or cyclopiazonic acid with ryanodine at inhibitory concentrations caused a significant increase in nuclear Ca^{2+} loading at low concentrations of extranuclear added Ca^{2+}. Our results show that the perinuclear lumen in embryonic chick ventricular

cardiomyocytes is capable of autonomously regulating nucleoplasmic Ca^{2+} fluxes.

Dedication

To Maria

Epigraph

"Nothing in the world can take the place of Persistence. Talent will not; nothing is more common than unsuccessful men with talent. Genius will not; unrewarded genius is almost a proverb. Education will not; the world is full of educated derelicts. Persistence and Determination alone are omnipotent. The slogan "Press On", has solved and will always solve the problems of the human race."

-Calvin Coolidge

Introduction

Ionized Ca^{2+} is able to function efficiently as a multifunctional cytosolic intracellular second messenger by virtue of the significant differences in Ca^{2+} concentration within and surrounding the cellular environment [Berridge, 1997; Bootman and Berridge, 1995; Carafoli et al., 1997]. Furthermore, Ca^{2+} is capable of performing a pivotal role in the regulation of many diverse nuclear processes. Nuclear Ca^{2+} signaling has been implicated in the control of 'immediate early/delayed-response' gene expression, cell division, protein import, and apoptosis via the activation of various Ca^{2+}-dependent endonucleases, proteases, kinases, and phosphatases [Bachs et al., 1992; Carafoli et al., 1997; Cruzalegui et al., 1999; Gilchrist et al., 1994; Hardingham et al., 1997; He et al., 1997; Johnson et al., 1997; Jones et al., 1989; Means, 1994; Qi et al., 1997]. It is becoming more widely accepted that Ca^{2+} ion concentrations in the nucleoplasm are regulated by specific Ca^{2+} transport mechanism, similar to those also found in other organelles within the cell. It performs this integral role because of the presence of necessary Ca^{2+} flux regulatory components consisting of Ca^{2+}-ATPases, RyR, inositol 1,4,5-trisphophate (IP_3) receptors, inositol 1,3,4,5-tetrakisphosphate (IP_4) receptors, and a number of Ca^{2+}-binding proteins found within the perinuclear lumen [Berridge, 1993; Divecha et al., 1993; Hagar et al., 1998; Koppler et al., 1993; Malviya et al., 1990; Pesty et al., 1998]. However, it is unclear how these different proteins and biochemical pathways function together and more importantly very little is currently known about the role that the nucleus plays in regulating these pathways. Early studies conducted by Al-Mohanna et

al. [al Mohanna et al., 1994] attempted to resolve the question of whether nuclear Ca^{2+} is autonomously regulated and demonstrated an attenuation of large cytosolic transients into the nucleus. The controversy of whether the nucleus is able to independently or dependently regulate large Ca^{2+} fluxes originating in the cytoplasm from being transduced into the cell nucleus via the nuclear envelope lies in the discrepancies and differing methodologies currently being used to characterize the involvement of the nuclear envelope in mediating these Ca^{2+} fluxes. These discrepancies were most prominent in several studies which have shown basal levels of nuclear Ca^{2+} were below, equivalent, or above that of cytosolic Ca^{2+} [Himpens et al., 1994; Ikeda et al., 1996; Williams et al., 1987; Williams et al., 1985]. Furthermore, the difficulties intrinsic with quantifying Ca^{2+} concentrations either by fluorescent dyes which are entirely dependent upon altered dye compartmentalization and sensitivity in differing proteinaceous environments [Perez Terzic et al., 1997b] or patch-clamp studies [Holmberg and Williams, 1990; Mak and Foskett, 1994] have not resolved the controversy of independent nucleoplasmic Ca^{2+} regulation. More recently, several studies have shown that the nucleus also possesses the necessary molecular machinery required for autonomously mediating Ca^{2+} fluxes at the level of the nuclear envelope [Gerasimenko et al., 1995; Hennager et al., 1995; Humbert et al., 1996; Lanini et al., 1992; Nicotera et al., 1990; Santella and Kyozuka, 1997; Stehno Bittel et al., 1995a]. The perinuclear luminal space can serve the function as the mobilizable Ca^{2+} store that maintains the flux of Ca^{2+} from the cytosol into the nucleoplasm [al Mohanna et al., 1994; Gerasimenko et al., 1995]. The central

hypothesis in this study is that the regulation of nucleoplasmic Ca^{2+} concentration occurs by the mobilization of perinuclear Ca^{2+} pools involving specific Ca^{2+} pumps and channels at both the inner and outer nuclear membranes.

Literature Review

The ability of Ca^{2+} to mediate biological processes has now become an integral factor in various areas of cell biology, biochemistry, physiology, and pathology. Ca^{2+} appears to function in quantal informational units within the cells. Sydney Ringer made the first discovery of Ca^{2+} salts being a crucial component in "Ringer's Solution" [Ringer, 1883]. He performed a series of rudimentary, yet elegant experiments that demonstrated the requirement for Ca^{2+} in maintaining cardiac contractility. The study showed that the Ca^{2+}-dependence of beating heart muscle, but did not indicate the site of action for Ca^{2+}. Following Ringer's studies, another discovery that greatly advanced the importance of Ca^{2+} for biological function was the identification of the role of Ca^{2+} in intracellular processes [Heilbrunn and Wiercinski, 1947]. Ca^{2+} could diffuse into the cytosolic space, access the myosin contractile machinery, and cause muscle shortening. The response of muscle shortening clearly indicated a requirement for Ca^{2+}, but still did not demonstrate the involvement of Ca^{2+} in this physiological response. An important corollary was the discovery that this involvement clearly showed that the enzymatic activity was associated with myosin and more importantly that Ca^{2+} was the factor responsible for its activation [Bailey, 1942]. A seminal leap in furthering the importance of Ca^{2+} in biological systems was demonstrated when the integral role of Ca^{2+} in various pathways no longer limited its study to the field of muscle research [Kanno et al., 1973; Miledi, 1973; Timourian et al., 1972]. These studies, occurring 20 years later, broadened the field of study with which Ca^{2+} was involved. The results demonstrated the importance of Ca^{2+} in

neurotransmitter release, exocytosis, and oocyte maturation. Regardless of cell type, it was becoming clear that a common requirement for Ca^{2+} was necessary for proper cellular functioning.

The specialization of cells in multicellular organisms necessitates an inherent cooperative division of labour. This requirement ensures the proper functioning of the organism as a whole. This co-operativity is accomplished via a complex signalling mechanism, which is required for the correct modulation and synchronization of biological activity to occur. A select group of chemicals have become the accepted components involved with these signalling mechanisms, of which Ca^{2+} is a member. The inherent chemical properties of Ca^{2+} have pre-determined the important evolutionary role it plays as an intracellular 2nd messenger. Under "normal" basal conditions, total free Ca^{2+} concentrations within the cytoplasm of eukaryotic cells are approximated to be in the high mM range. If Ca^{2+} is to function effectively and efficiently as a chemical messenger, it becomes critical that the free Ca^{2+} concentrations be greatly reduced. This principle is an inherent biological requirement when viewed from an energetic perspective. The reduction of intracellular Ca^{2+} concentration would otherwise consume excessive amounts of energy.

1. Cytoplasm

Ubiquitously available biological compounds are present that are capable of buffering Ca^{2+}, so as to lower total free Ca^{2+} concentrations, but unfortunately they do so with either low affinity or low specificity. In order to achieve ideal conditions that permits Ca^{2+} to function as an intracellular 2nd messenger,

alterations were required that effectively reduce free Ca^{2+} concentrations to between μM and high nM levels. This is accomplished by various proteins that possess a peculiar conformational structure, which allows for high capacity binding. Even more importantly is the characteristic that they possess high specificity and perform binding in a reversible manner and in an environment where concentrations of competing ions may be orders of magnitude higher than that for Ca^{2+}.

1.1. Ca^{2+}-Binding Proteins

The ability of Ca^{2+} to serve as a messenger is determined primarily by its ionic and chemical properties [Carafoli and Penniston, 1985]. One of these properties is the ionic charge, which in turn defines its physical dimensions. Curiously, in a system of very strictly regulated biological processes, which seemingly involved very precise interactions with Ca^{2+}, it is the irregularly formed binding cavities for Ca^{2+} that predominate [Swain et al., 1989]. If one was to assume that perfect octahedral conformations of the ligand binding cavity was required in typical Ca^{2+}-binding proteins, as would be the case with Mg^{2+} serving as the ligand, it would inadvertently place to great a strain on the structural backbone of the involved proteins because of the smaller ionic radius [Jaiswal, 2001]. Fortunately, the propensity for proteins to form these perfect binding cavities is rare. In fact, it is these imperfect octahedral conformation that are the most abundant; thus, favouring Ca^{2+} as the ligand of choice. Another key factor that supports Ca^{2+} as the ideal messenger relies on the large concentration differential of Ca^{2+} from the extracellular vs. intracellular spaces. In most eukaryotic systems, this difference is measured as high as mM vs. sub-μM,

respectively [Barry and Bridge, 1993; Carafoli, 1988; Missiaen et al., 1992]. The implication of this polar distribution of Ca^{2+} concentration depends on the integrity of the plasma membrane and its ability to maintain an impermeable barrier to Ca^{2+}. Conversely, this situation allows for very little energy expenditure with which the cell must deal in order to transport Ca^{2+} from extracellular to intracellular spaces. The presence of large, relatively unlimited pools of Ca^{2+} readily available to flow naturally down its concentration gradient serves as a mechanism with which to induce and regulate potentially large fluctuations in intracellular Ca^{2+} concentrations [Barry and Bridge, 1993; Himpens et al., 1995]. With regards to energy considerations, namely adenosine 5'-triphosphate (ATP) consumption and the liberation of inorganic phosphate as a by-product, it is very likely that large uncontrolled influxes of Ca^{2+} may cause the formation of Ca^{2+}-phosphate crystals. These crystals would eventually precipitate within the cell and subsequently cause damage and death [Misra, 2000]. Hence, this is another advantage that would favour the maintenance of low homeostatic Ca^{2+} concentrations within the intracellular space.

As previously described, there is a requirement for proteins that are capable of interacting in a specific manner with Ca^{2+}, in order to ensure the proper functioning of biological systems. The required characteristics involve proteins that are capable of interacting with high specificity, affinity, and in a reversible manner. These characteristics allow for the proper regulation of intracellular concentrations so that they are maintained and that the modulation of spatio-temporal signalling of Ca^{2+} is made possible. These proteins exist in

several proto-typical groups that either regulate cellular Ca^{2+} homeostasis or propagating informational signals to the appropriate targets. The proteins that are primarily responsible for sensing the intracellular Ca^{2+} concentration and can be characterized by a binding structure traditionally referred to as the EF-hand. After binding Ca^{2+}, this motif transduces an informational signal through a structural alteration in the proteins quaternary structure. This then allows the protein to now accept interactions with target effector proteins such as enzymes for example.

1.1.1. EF-Hand

The EF-hand superfamily of proteins is typically defined by initial characterization studies performed on parvalbumin [Kretsinger, 1972; Moews and Kretsinger, 1975]. These proteins have been categorized into 66 subfamilies with approximately 660 member proteins. The general structural motif is described as 2 perpendicular helices interrupted by a loop structure. This helix-loop-helix module is normally found in groups of 2 or 4 together and the sequence conservation at the loop does not tolerate a high range of variability. The Ca^{2+} ion will bind within this imperfect pentagonal bi-pyramidal cavity via a constellation of 7 oxygen atoms from a set of amino acids comprised of Asp, Asn, Glu, Thr, and Ser side chains, backbone carbonyls, or from intermediary water molecules. The ligand interaction in the loop structure is normally dependant upon the residues in position 1, 3, 5, 7, 9, and 12. The predominant residue in position 1 is Asp, with Glu in position 12. The commonality of these features make it likely that these proteins are derived from an evolutionarily

conserved common ancestral gene family. Ca^{2+}-binding affinities for parvalbumin have been reported at K_d values ranging from 0.1 to 4 μM, which are dependent upon Mg^{2+}, pH, or ionic strength [Donato, 1986; Heizmann, 1984]. It is widely accepted that the sole function for parvalbumin is to mediate Ca^{2+} fluctuations within the cell by buffering Ca^{2+} concentrations within the cytosol. The initial evidence of parvalbumin being able to function in this role are observations localizing this protein to fast-twitch fibers [Inaguma et al., 1991; Leberer and Pette, 1986; Stuhlfauth et al., 1984]. Currently, direct evidence has confirmed that parvalbumin is actively involved in mediating Ca^{2+} fluxes [John et al., 2001; Lannergren et al., 1993; Muntener et al., 1995].

Another important set of proteins that possess EF-hand Ca^{2+}-binding domains, are those that also possess well known enzymatic activities. Hence, the EF-hand sequence is concatenated with its encoded functional, enzymatic domain. Generally, binding of Ca^{2+} to these proteins allows for subsequent interactions with its downstream targets so as to communicate the informational signal encoded by the Ca^{2+}-binding. These targets are commonly enzymes, but can also bind structural proteins and DNA. Calmodulin is one example. Briefly, the structure of calmodulin is described by 2 terminal domains, each binding 2 Ca^{2+} ions and is separated by an imperfectly formed α-helix [Babu et al., 1985; Barbato et al., 1992; Heidorn and Trewhella, 1988]. Upon Ca^{2+}-binding, the calmodulin protein will then be capable of interacting with proteins possessing appropriate calmodulin-binding domains. Binding takes place at the central helix, where calmodulin subsequently collapses around the target [Meador et al., 1993;

Osawa et al., 1999]. This interaction allows the target protein to alter its structure; thereby, becoming activated. Calmodulin binding affinity to its various targets is also dependent upon its phosphorylation status, which is modulated by casein kinase II [Meggio et al., 1987; Nakajo et al., 1988; Quadroni et al., 1994; Sacks et al., 1992] and various tyrosine kinases [Benguria et al., 1994; Colca et al., 1987; Corti et al., 1999; Fukami et al., 1986; Joyal and Sacks, 1994; Sacks et al., 1992; Sacks and McDonald, 1988]. *In vitro* modification by casein kinase II and tyrosine kinases either inhibit [Quadroni et al., 1998] or activate [Corti et al., 1999] calmodulin binding affinity to its enzyme targets. The interaction of Ca^{2+} via EF-hands is crucial in determining cellular viability by allowing for the proper decoding and translation of spatiotemporal Ca^{2+} fluctuations.

1.1.2. Non-EF-Hand

In addition to EF-hand Ca^{2+}-binding proteins, there are various proteins responsible for mediating the Ca^{2+} homeostasis, which are predominately non-EF-hand Ca^{2+}-binding proteins. They generally do not transduce a Ca^{2+} signal to other targets, but are not excluded from deciphering Ca^{2+} signals. These binding motifs are quite varied in both structural and functional characteristics, but the most abundant modules are the C_2 domain, annexins, and gelsolin binding domains. Characteristically, these proteins exhibit at a lower affinity for Ca^{2+} than traditional EF-hand proteins. They are localized to the lumen of various organelles and membranous systems, as opposed to the intracellular nature of EF-hand proteins. The C_2 domains are primarily associated with phosopholiapase C (PLC), protein kinase C (PKC), and cytosolic phospholiapase

A_2 (cPLA2). This domain is responsible for the Ca^{2+}-dependant localization of these proteins to membrane fractions. Their structure consists of compact ß-sheets that provide an incomplete coordination cavity for the binding of Ca^{2+}, which is completed by phospholipids [Sutton et al., 1995]. It has been proposed that the C_2 domains translate the Ca^{2+} signal by altering its electrostatic potential, thereby functioning as a switch instead of undergoing a conformational change [Shao et al., 1997]. Another non-EF-hand Ca^{2+}-binding protein family is the annexins. The biological significance of this family of proteins is currently ambiguous. Its structure is generally described as being comprised of five α-helices formed from four highly conserved repeats of 70 amino acids, which in turn generates a right-handed super-helix [Brisson et al., 1991; Burger et al., 1996; Huber et al., 1992; Mosser et al., 1991]. Its Ca^{2+}-binding sites have been attributed to its membrane binding domain called the endonexin fold [Geisow et al., 1986; Kretsinger and Creutz, 1986]. The annexins are generally characterized by their ability to reversibly associate with negatively charged phospholipids in a Ca^{2+}-dependant manner. Thus, Ca^{2+} is able to mediate annexin's ability to mediate vesicular membrane trafficking, ion channel activity and PLA2 activity. An additional non-EF-hand Ca^{2+}-binding protein is gelosin. It exists as two differentially localized species, either in the intra- or extracellular space [Kwiatkowski et al., 1986]. The intracellular variant mediates cellular motility. The extracellular isoform scavenges actin inadvertently released into the extracellular space during cell death, so as to prevent polymerization and alterations in blood viscosity. Structurally, it is comprised of six 120 to 150 amino

acid repeats [Kothakota et al., 1997]. The gelsolin binding domain regulates the binding, cutting, and capping of actin filaments in a Ca^{2+}-dependant manner [Yin and Stossel, 1979].

As well as intracellular localized proteins, there are a number of proteins residing within the lumen of various organelles that function as Ca^{2+}-storage proteins; such as, calsequestrin and calreticulin. They are normally characterized by the ability to reversibly bind Ca^{2+} with low affinity but very high capacity. They function to maintain a cellular Ca^{2+} concentration within a viable homeostatic range. Calsequestrin is the major Ca^{2+}-binding protein to reside within the sarcoplasmic reticulum. As a consequence of the conformational alteration that calsequestrin undergoes as a result of Ca^{2+}-binding, it may also indirectly affect the ryanodine receptor Ca^{2+} flux activity [Beard et al., 2002; Glover et al., 2001; Murray and Ohlendieck, 1998; Szegedi et al., 1999]. This interaction may be tightly regulated and describes an efficient mechanism by which Ca^{2+} is capable of dynamically regulating its own intracellular Ca^{2+} concentration. Calreticulin is an analogous protein to calsequestrin, but resides in the endoplasmic reticulum. Its primary function is to serve as a storage container for Ca^{2+}, but it is also capable of Ca^{2+}-dependant lectin-like chaperone activity [Baksh and Michalak, 1991; Bergeron et al., 1994; Li et al., 1998; Tjoelker et al., 1994].

2. Plasma Membrane

In conjunction with the afore mentioned proteins serving to buffer Ca^{2+} and maintain cellular Ca^{2+} homeostasis, proteins intrinsically associated with

membranes, are generally responsible for regulating free cytosolic Ca^{2+} concentrations. The membrane transport systems are generally localized to the plasma membrane, inner mitochondrial membrane, sarco/endoplasmic reticulum, and nuclear membranes. The plasma membrane possesses several pumps and channels, responsible for the efflux or influx of Ca^{2+} in the cell, respectively. The pumps fall into 2 categories: either low affinity, high capacity like the Na^{+}/Ca^{2+} exchanger, or high affinity, low capacity like the Ca^{2+}-ATPase. Conversely, the family of Ca^{2+} channels are numerous and greatly variable. Either by Voltage, receptors, or store interactions can regulate these channels to control the inward flux of Ca^{2+}. Following this influx and subsequent transduction of Ca^{2+} signals via the increase in intracellular free Ca^{2+}, a removal of this Ca^{2+} is necessary to prevent any potential damage caused by Ca^{2+}-overload.

2.1. Ca^{2+} Channels

As previously mentioned, these membrane channels are divided into 3 major groups; voltage-operated, receptor-operated, and store-operated channels. The voltage-operated channels are the most extensively studied, typical of excitable tissues, and are controlled via membrane depolarization. These channels are normally characterized by high specificity. Within this group, these channels are typically divided into two subclasses: 1) T-type and 2) L, N, P, R, and Q-type Ca^{2+} channels, based upon physiological and pharmacological properties. The best known are the L-type channels, which are dihydropyridine sensitive, high voltage sensitive, with a relatively brief duration open time. N-type channels are dihydropyridine insensitive. Generally, all voltage-operated channels are structurally similar and consist of 5 subunits. The receptor-

operated channels are generally activated by a ligand like L-glutamine. The activation usually occurs via 2 types; ionotropic receptors and metabotropic receptors. Ionotropic receptors are characterized by their ability to regulate a direct influx of Ca^{2+} into the cell. The ionotropic receptors are divided into 3 groups; kainite (KA), α-amino-3-hydoxy-5-methyl-4-isoxazole propionate (AMPA), and N-methyl-D-aspartate (NMDA). These receptors ultimately function to mediate Ca^{2+} influx into the postsynaptic neuron. The store-operated channels seemingly function via a compensatory mechanism, which are triggered to open when intracellular Ca^{2+} storage sites are depleted. As of yet, the mechanism by which intracellular storage compartments relay their state of filling and signal the activation of an inward Ca^{2+} current is unknown, but is generally referred to as capacitive Ca^{2+} entry. This mechanism has been proposed to operate in either a direct or indirect interaction of the endoplasmic reticulum with the plasma membrane resident channels. These interactions have been proposed to occur either through an unidentified factor released from the endoplasmic reticulum, or through physical contact of the endoplasmic reticulum with the channels, respectively.

2.2. Ca^{2+}-ATPase

The plasma membrane Ca^{2+} ATPase pump (PMCA) is responsible for the removal of large quantities of intracellular Ca^{2+}. In most non-excitable cell types, it does not comprise a large quantitative portion of the plasma membrane, but is still able to function as the central factor in modulating free intracellular Ca^{2+} concentration. Mechanistically, it functions along the same lines as the SERCA pump. They can be differentiated not only by their cellular localization, but also

by their differences in Ca^{2+} transport stoichiometry. SERCA pumps normally exhibit a 2:1 stoichiometry in Ca^{2+} ions transported: ATP hydrolysed vs. the 1:1 stoichiometry found in PMCA pumps. Regulatory factors specifically affect the Ca^{2+} affinity of the PMCA pump, where the K_d varies between ~20 μM to less than 1 μM in resting and increased coupled interactions with different compounds.

2.3. Na^+/Ca^{2+} Exchanger

Another protein localized to the plasma membrane and involved in mediating Ca^{2+} homeostasis in excitable cells is the Na^+/Ca^{2+} exchanger. The importance of Na^+ was first documented when it was observed that it could affect cardiac contractility. The importance of the exchanger was not revealed until studies demonstrated that Ca^{2+} efflux was Na^+-dependent. Furthermore, it involved a carrier that exchanged Na^+ for Ca^{2+} [Baker et al., 1969; Blaustein and Hodgkin, 1969; Reuter and Seitz, 1968]. It was first cloned in 1990 [Nicoll et al., 1990] and was characterized as a protein with 990 amino acids and a molecular mass of 120 kDa. Generally, its structure has 11 transmembrane domains, where the Ca^{2+} regulatory and transport sites are separately localized within the protein. Its activity is modified by phosphatidyl-inositol 4,5-bisphosphate (PIP_2), which strengthens its involvement in mediating cellular Ca^{2+} signalling [Hilgemann and Ball, 1996]. The fundamental properties of the exchanger are that: 1) Ca^{2+} is extruded against its concentration gradient, where the energy is provided by the simultaneous influx of Na^+, 2) its stoichiometry is 3:1 [Reeves and Hale, 1984], 3) the exchanger activity is dependent upon membrane potential, and 4) it is reversible. It is capable of very high turnover rates, which

have been recorded as high as 10x greater than that of the PMCA [Barry et al., 1986; Cannell, 1991], but does so at a relatively low affinity for Ca^{2+} [Hilgemann, 1990; Hilgemann et al., 1991]. With reported K_m values ranging from 1 to 10 μM, it may appear to be ineffective at clearing increases in Ca^{2+} concentrations, hence disproving its importance in maintaining Ca^{2+} homeostasis. Conversely, studies have proposed that the exchanger is sensing a localized microdomain of highly elevated Ca^{2+} [Frank et al., 1992], thus supporting the exchanger's role in Ca^{2+} homeostasis.

3. Intracellular Organelles

The main intracellular Ca^{2+}-storage compartment found in eukaryotic cells is the membranous lattice structure called the endo/sarcoplasmic reticulum. To function as a reservoir for a readily available source of intracellular Ca^{2+}, it must possess 3 key components; a method of moving Ca^{2+} into the lumen of the endo/sarcoplasmic reticulum, a means of sequestering Ca^{2+} to increase storage capacity, and potentially affect ligand-gated channels, and a mechanism to release Ca^{2+} on demand from this reservoir. The main components capable of meeting these requirements are the SERCA pump, calsequestrin, and the RyR. This reservoir of Ca^{2+} has been measured by various methods and total Ca^{2+} concentrations have been estimated to be within the high mM Ca^{2+} range.

3.1. Endoplasmic reticulum/Sarcoplasmic reticulum (ER/SR)

Ca^{2+} release from the endo/sarcoplasmic reticulum is achieved via 2 major channel families; RyR and IP_3 receptors. Both of these channels are coexpressed in various cell types and it appears that different combinations may be responsible for varying functional characteristics. Each of the channels is

differentially activated. IP_3 receptors are ligand mediated; such that, activation only occurs after an appropriate interaction with IP_3. RyR activation can be regulated, via membrane depolarization or by interaction with an intracellular 2nd messenger such as (oxidized nicotinamide-adenine dinucleotide (NAD+) or cyclic adenosine diphosphate ribose (cADPr).

3.1.1. IP_3

The importance of IP_3 as a key 2nd messenger mediating Ca^{2+} fluctuation was first discovered in 1975 [Michell, 1975]. He observed that as a consequence of phosphatidyl-inositol (PI) exposure, a cytosolic increase in Ca^{2+} was induced. Subsequently, Berridge and co-workers were able to resolve that the fundamental component responsible for the increase observed by Michelll was in fact caused by IP_3. Furthermore, the source for this pool of releasable Ca^{2+} was non-mitochondrial and originated from the endoplasmic reticulum. The basic mechanism of IP_3 generation involves the activation of PI-specific PLC via 1st messengers; such as, ATP, platlet-derived growth factor (PDGF), epidermal growth factor (EGF), etc. Upon activation, PI, phosphatidyl-inositol 4-phosphate (PIP1), or PIP2 is then cleaved to generate diacylglycerol (DAG) and IP_3 via PLC. The IP_3 released is now free to migrate or diffuse throughout the cell and exert its effect via an IP_3 receptor localized to the endoplasmic reticulum, plasma membrane, or the nuclear envelope. Five variant isomeric types are known to exist and this variation may be responsible for differences in temporal Ca^{2+} fluctuations in a wide selection of cell types. Structurally, the IP_3 receptor appears to possess a four-fold symmetry, with a central pore. The amino acid

sequence of the monomer shows that the receptor possesses 3 domains: a large N-terminal sequence responsible for IP_3-binding, a central regulatory domain, and a short transmembrane anchoring C-terminal domain. It is proposed that binding of IP_3 to the N-terminus induces a structural change in the C-terminus portion, which then opens the Ca^{2+} channel. Ca^{2+} channel activity can also be mediated by other factors like Ca^{2+}, calmodulin, and ATP. Ca^{2+} also has an effect upon the cytosolic and luminal face of the IP_3 receptor. Luminal modulation of IP_3 receptor-mediated Ca^{2+} channel activity derives from a structural feature on the luminal face, which may also function as a Ca^{2+}-binding site. This modulation presents itself as a consequence of relatively low levels of filling of the endo/sarcoplasmic reticulum storage compartment, which alters the sensitivity of the receptor to mediate Ca^{2+} release via IP_3 ligand binding. Conversely, Ca^{2+} effects upon the cytosolic face of the receptor are primarily biphasic; thus, implicating a stimulatory and inhibitory binding site for modulating Ca^{2+} channel activity.

Another factor that may affect the potentiation of Ca^{2+} release by Ca^{2+} is the immunophilins, specifically FK506 binding protein 12(FKBP12). Studies have shown that IP_3 receptors and FKBP12 colocalize and the latter may function to stabilize the quaternary structure of the receptor. Instability may occur when IP_3/FKBP12 interactions are interrupted by FK506. FK506 induces a leak state on the receptor. Studies have shown that a very similar interaction also occurs in ryanodine receptors and FKBP12.

3.1.2. RyR

The ryanodine receptor was originally discovered and localized to the sarcoplasmic reticulum of skeletal and cardiac tissues. The receptor possesses a biphasic activation/inactivation Ca^{2+} channel activity. Relatively high Ca^{2+} concentrations inhibits Ca^{2+} release; conversely, low concentrations induces the receptor to exist in a refractory state of flux. The receptor exists in 3 tissue specific isoforms; RyR1 in skeletal, RyR2 in cardiac and brain, and RyR3 in cerebellum. The 3 Ca^{2+}-binding regions reside in the N-terminal domain. There are calmodulin-binding sites near the channel pore. Calmodulin directly effects channel opening in a Ca^{2+}-dependant manner. Increased binding of calmodulin at low Ca^{2+} concentrations potentiates the opening of the channel to Ca^{2+}. A reduction in calmodulin-binding will be the result in the presence of higher Ca^{2+} concentrations. ATP also affects channel flux by increasing the receptor's affinity for Ca^{2+}. Another key regulator of channel activity is FKBP12. It functions in a manner similar to that of IP_3 receptors. The increase in stability shifts the receptor to a zero conductance state; likely, through ameliorating the interaction between the 4 subunits of the channel. The physiological significance of the RyR, with respect to Ca^{2+} signalling, involves cellular processes such as capacitative Ca^{2+} entry and Ca^{2+} induced Ca^{2+} release. The process of capacitative Ca^{2+} entry in skeletal muscle is in part mediated by the ryanodine receptor. The mechanical coupling involved during this process begins with a depolarizing potential that travels down the transverse tubule to activate L-type voltage channels, dihydropyridine receptors (DHPR). Either through a direct or indirect interaction between DHPR and RyR, a signal is decoded that opens the

Ca^{2+} release channel. A protein that has been implicated in being an intermediary member of this heteromeric protein-complex is triadin [Flucher et al., 1993; Groh et al., 1999; Protasi et al., 2000; Takekura and Franzini-Armstrong, 1999; Tanaka et al., 2000].

Another important mechanistic paradigm that involves the release of Ca^{2+} from RyR is now commonly known as Ca^{2+} induced Ca^{2+} release (CICR). CICR is a process by which μM concentrations of Ca^{2+} in the cytoplasm induce the opening of SR Ca^{2+} release channels. However, this process is unlikely to function in skeletal muscle because of the high Ca^{2+} concentrations required to induce a Ca^{2+} release event. On the other hand, it may participate in propagating Ca^{2+} signals instead of serving as the source of the signal. In cardiac tissues, CICR is the prime stimulus for Ca^{2+} release.

3.1.3. SERCA

Within the ER/SR, the component attributed to pumping Ca^{2+} into the lumen is the Ca^{2+}-dependent ATPase. As previously mentioned, the SERCA pump possesses a 2:1, Ca^{2+}:ATP stoichiometry. Antagonists such as thapsigargin [Thastrup et al., 1990], cyclopiazonic acid (CPA) [Kurebayashi and Ogawa, 1991; Seidler et al., 1989], or t-butyl hydroquinone [Dolor et al., 1992; Foskett and Wong, 1992] can inhibit ATP stoichiometry and pump activity. The stoichiometry of the pump has been confirmed by crystallization of the protein. The pump is divided into 3 tissue specific groups and subtypes; SERCA1a and SERCA1b are adult and neonatal muscle isoforms [Brandl et al., 1987], respectively, SERCA2a and SERCA2b are endoplasmic reticulum resident

specific to muscle or varying non-muscles [Campbell et al., 1992; Eggermont et al., 1989; Lytton and MacLennan, 1988], respectively, and SERCA3 prominently found in platelets [Bobe et al., 1994; Burk et al., 1989; Wuytack et al., 1994]. All of the variations present in the SERCA protein family are generated from 3 genes that are alternatively spliced [Grover and Khan, 1992].

The most widely studied regulatory mechanism, other than that provided by transcriptional regulation, is the interaction provided by phospholamban [Simmerman and Jones, 1998]. This interaction inhibits SERCA catalytic activity by decreasing the pump's affinity for Ca^{2+}. Studies attempting to further our understanding, involve experiments determining whether a "cross-talk" molecular mechanism exists between SERCA and RyR [Gilchrist et al., 1992]. This proposed interaction would allow the pump to sense an inhibitiory feedback signal in the event of RyR Ca^{2+} channel closure.

The initial Ca^{2+} movement that occurs immediately after Ca^{2+} channel opening at either the plasma membrane or the endoplasmic reticulum can be visualized as puffs or sparks, which are generated from IP_3 or RyR, respectively [Cheng et al., 1993; Yao et al., 1995]. These confined spatial events occur in a very short time-scale. They are observed before these highly localized Ca^{2+} concentrations diffuse throughout the cytoplasm. If these elementary release events occur on a large enough scale and in near enough vicinity to neighbouring channels, the initial Ca^{2+} spark or puff can become propagated throughout the cell [Berridge, 1997; Berridge et al., 1998]. The propagation is also conditional upon the presence of a threshold concentration of IP_3 or Ca^{2+} at the appropriate

receptor. Therefore, a Ca^{2+} wave is generated, transforming a local event into a global response so as to communicate information to relatively distal regions of the cell. Ca^{2+} waves appear with varied spatio-temporal characteristics [Jaffe, 1993; Jaffe, 1995], which may induce different types of physiological responses that are to be coordinated throughout the cell. Another type of global informational signalling mechanism operating within cells is Ca^{2+} oscillations [Woods et al., 1987]. These periodic fluctuations are normally associated with plasma membrane Ca^{2+} channels being opened due to rhythmic changes inherent in muscles. Recently, another type of oscillation has been characterized that appear to be generated at the level of the endo/sarcoplasmic reticulum. The propagation of these oscillations is generally accomplished via CICR [Hua et al., 2000; Jaffe, 1993]. The physiological relevance of this Ca^{2+} phenomenon may be to differentially transmit a signal to various components within the cell. These various components may be sensitive to different Ca^{2+} concentrations. Ca^{2+} oscillations allow for an intermittent binary-like signalling mechanism to operate. The activation of different components with relatively high amplitudes of Ca^{2+} prevent the whole cell from being dangerously exposed to prolonged, elevated Ca^{2+} concentrations. These Ca^{2+} oscillations may impact the genetic programming of the cell; thereby, affecting the regulation of genetic expression in a Ca^{2+}-dependant manner at the level of the nucleus [Dolmetsch et al., 1998; Hu et al., 1999]. For example, a direct correlation between IP_3 mediated Ca^{2+} oscillations and alterations in NFAT gene expression have been demonstrated [Li et al., 1998]. The importance of Ca^{2+} waves is not constrained only to one cell

but can also migrate from one cell to the next via gap junctions. This allows for a cooperativity and coordination that is essential in the functioning of organs; such as, peristalsis or the coordinated beating of the heart.

3.2. Mitochondria

Mitochondria were long perceived as performing the single, but important task of being the primary generators of ATP within most eukaryotic cells. It was generally accepted that this was the only functional role that mitochondria played. It was originally hypothesized that mitochondria were derived from prokaryotes that entered into a symbiotic relationship with eukaryotes [Gray, 1998; Margulis, 1996]. Structurally, two highly specialized membranes define mitochondria. These membranes delineate and segregate the mitochondrial lumen called the matrix, a narrower intramembrane space, and the outer membrane. Fractionation of mitochondria into these separate components demonstrates the unique composition of proteins in each compartment.

The significant discovery of the chemiosmotic theory of oxidative phosphorylation in mitochondria immediately brought attention to the importance of these organelles in cellular function [Mitchell and Moyle, 1967]. This core discovery explained why oxygen is necessary for life, and how this relatively simple element is required for the generation of ATP. ATP is the major energy currency utilized in most organisms, where 80-90 % of all ATP is generated at the mitochondria.

Besides the central function of mitochondria to provide ATP, they are also capable of sequestering Ca^{2+}. As with other cellular Ca^{2+} storage systems,

mitochondria possess both uptake and efflux pathways which are responsible for the proper regulation of Ca^{2+} sequestration.

3.2.1. Ca^{2+} Uptake

Mitochondrial Ca^{2+} uptake is governed by principles of the chemiosmotic theory. Accumulation of Ca^{2+} can occur at the expense of ATP hydrolysis or via the energy provided by substrate oxidation. The fundamental mechanism operates via an electrochemical potential gradient formed across the inner mitochondrial membrane. H^+ extrusion provides the driving force for uptake. Hence, the resulting Ca^{2+} gradient is mediated by kinetics and not by thermodynamics [Azzone et al., 1977]. Ca^{2+} uptake occurs via two electrogenic pathways and is susceptible to inhibition by ruthenium red [Crompton and Andreeva, 1994; Litsky and Pfeiffer, 1997; Matlib et al., 1998].

The first uptake pathway was discovered and attributed to a poorly characterized and as of yet unidentified protein called the uniporter [Dawson et al., 1971; Wingrove et al., 1984]. The uniporter possesses both activation and transportation sites [Bygrave et al., 1971; Gunter and Pfeiffer, 1990; Scarpa and Azzone, 1970; Vinogradov and Scarpa, 1973]. It normally functions by being activated by Ca^{2+} and transporting it down its electrochemical gradient into the mitochondrial matrix during conditions of elevated cytosolic Ca^{2+} concentrations [Kroner, 1986; Saris and Kroner, 1990]. It is regulated by divalent cations, nucleotides, and is sensitive to ruthenium red inhibition [Drahota et al., 1969; Reed and Bygrave, 1974; Rossi et al., 1973; Vainio et al., 1970]. Regulation is mediated by metal binding sites that modulate affinity for Ca^{2+}. Magnesium can

decrease the Vmax and increase the Km of the uniporter. Mg^{2+} causes this effect by binding to regulatory sites and thereby not occluding the Ca^{2+} binding transporter sites [Bragadin et al., 1979].

The second form of mitochondrial uptake that has recently been discovered in liver is called the RaM (rapid uptake mode) [Sparagna et al., 1994; Sparagna et al., 1995]. This form of uptake is characterized by a very high rate of uptake, which is quickly inhibited after its initiation [Sparagna et al., 1995]. It operates as a uniporter and relies upon the electrochemical gradient as the driving force, which was demonstrated by inhibiting the uptake by exposing mitochondria to an uncoupler [Sparagna et al., 1995]. Studies comparing uptake rates between the uniporter and RaM at its lowest activity have indicated that RaM Ca^{2+} uptake is at least 300 times that of the uniporter [Brahm, 1977; Gunter and Pfeiffer, 1990]. Mitochondrial RaM is more efficient when it is exposed to pulsatile Ca^{2+} transients of ~400 nM, as opposed to exposure to steady-state Ca^{2+}. This uptake pathway is also inhibited by ruthenium red [Sparagna et al., 1995]. It has been suggested that both the uniporter and RaM are the same protein complex, but in different conformational states [Mironova et al., 1994; Mironova et al., 1982; Saris et al., 1993; Sottocasa et al., 1972; Zazueta et al., 1991; Zazueta et al., 1998].

One possible role for Ca^{2+} uptake would be to mediate energy metabolism. It would function to modulate respiration by cytosolic Ca^{2+} fluctuations, where increases in energy demand are accompanied by increases in cytosolic Ca^{2+} concentration. This was demonstrated in cardiac cells, where

impairments in cytosolic Ca^{2+} signalling adversely affected their ability to properly regulate their energy metabolism [Di Lisa et al., 1993].

3.2.2. Ca^{2+} Efflux

Theoretically, with cytosolic Ca^{2+} concentrations generally measured at 0.1 μM and mitochondrial membrane potential at –180 mV, Ca^{2+} concentration within the matrix should be 0.1 M [Rizzuto et al., 2000]. This poses a rather dangerous situation where Ca^{2+} precipitates can form, thereby damaging the organelle. Hence, a mechanism must exist that allows for the removal of Ca^{2+} from the mitochondrial matrix via an efflux pathway.

Ca^{2+} efflux pathways occur either through Na+ dependent/independent mechanisms or via the permeability transition pore (PTP). After uniporter inhibition by ruthenium red, efflux can be stimulated by Na^+ [Crompton et al., 1978]. The Na^+-dependent efflux pathway occurs via the Na^+/ Ca^{2+} exchanger, which has been implicated in physiological Ca^{2+} cycling. It is strongly inhibited by Sr^{2+} [Saris and Bernardi, 1983]. Efflux transport stoichiometry is $3Na^+$:1 Ca^{2+} [Jung et al., 1995; Wingrove and Gunter, 1986]. Conversely, the Na^+-independent pathway exhibits a stoichiometry of $2H^+$:1 Ca^{2+} [Gunter et al., 1991; Gunter et al., 1983]. Another component that may be involved is the mitochondrial permeability transition pore. It is characterized by a maximal conductance of 1200 pS with a corresponding pore of ~2 nm [Crompton and Costi, 1990]. This efflux pathway most likely functions under conditions where large intramitochondrial Ca^{2+} fluxes with an accompanying ATP/ADP ratio decrease [Zoratti and Szabo, 1995]. Whether this pathway functions under

physiological conditions is still fairly uncertain [Bernardi and Petronilli, 1996], but recent studies have implicated its involvement in CICR [Ichas et al., 1997].

Mitochondria may sequester Ca^{2+} to act as a Ca^{2+} buffering storage system. Initially, the level of mitochondrial Ca^{2+} accumulation was not thought to be not significant. This was disproved by the utilization of targeted aequorin [Rizzuto et al., 1992] that allowed for the direct measurement of mitochondrial Ca^{2+} concentration. They were able to demonstrate that Ca^{2+} released from ER caused a subsequent increase in mitochondrial matrix Ca^{2+} concentration. This implied that mitochondrial Ca^{2+} uptake invariably followed cytoplasmic Ca^{2+} fluctuations induced by an agonist. Further work demonstrated that the uniporter was responsible for the accumulation. Furthermore, the uptake was sensitive to ruthenium red and proton motive force (PMF) collapse [Rizzuto et al., 1993]. Initial studies demonstrated that fluctuations in Ca^{2+} concentration were translated into oscillatory NADH changes [Pralong et al., 1994]. These results were shown to be the result of Ca^{2+} oscillations originating from the mitochondrial matrix [Hajnoczky et al., 1995]. These studies indicate that Ca^{2+} fluctuations can encode an informational signal that can be interpreted by mitochondria as a need to increase its metabolism, resulting in increased ATP production [Jouaville et al., 1999]. Mitochondrial metabolism is functionally coupled to Ca^{2+} signalling, where studies demonstrate that mitochondria are capable of responding to increased energy load demands [Hajnoczky et al., 1999; Jouaville et al., 1999; Robb-Gaspers et al., 1998].

3.3. Nucleus

The nucleus and its membranous barrier, the nuclear envelope surround and segregate the cells genetic material, thus, defining the boundaries between the cytoplasm and the nucleoplasm. Another key feature is the nuclear pore complex, which perforates the nuclear envelope. Generally, the nucleus is a container for genetic material, where the decoding and the translation of DNA was performed. Also, the mechanisms involved in the shuttling of proteins between the cytoplasm and the nucleoplasm have been of great interest. It was not until recently that investigations implicated the nucleus as being able to autonomously regulate Ca^{2+} fluctuations.

3.3.1. Nuclear Envelope

The nuclear envelope is comprised of two membranes: the outer nuclear membrane that faces the cytoplasm and is contiguous with the ER; and the inner nuclear membrane that faces the nucleoplasm, and is closely associated with the nuclear lamina. The inner and outer membranes define a narrow luminal space called the perinuclear lumen. It was originally proposed that the inner and outer nuclear membranes were not distinct [Schindler et al., 1985]. However, it was determined that proteins localized to the inner membrane did not migrate to the outer membrane or the ER [Gant and Wilson, 1997].

The ability of nuclei to sequester Ca^{2+} was convincingly demonstrated; indicating that the molecular machinery required to maintain a storage compartment for Ca^{2+} is present in the nuclear envelope. This storage compartment resides in the space between the outer and inner nuclear envelopes and is termed the perinuclear cisternal space or lumen, which appears

to be contiguous with the lumen of the endoplasmic reticulum. Studies have shown that this compartment is dynamic and that Ca^{2+} can be released into the nucleoplasm by challenging nuclei with IP_3 or cADPr. Both release channels have so far been localized to the inner nuclear envelope, while the SERCA-like pump and an IP_4 receptor is present in the outer nuclear membrane. Several studies have demonstrated that each of these components are fully functional and appear to be very similar to their counterparts in the endo/sarcoplasmic reticulum. One of these key systems is a nuclear-resident phosphoinositide enzymatic pathway.

The components required for the generation of IP_3 have been localized to the nucleus [Smith and Wells, 1983a; Smith and Wells, 1983b; Smith and Wells, 1984]. Even more intriguing is the potential that the nuclear inositol lipid cycle is independently regulated [Avazeri et al., 1998; Humbert et al., 1996; Lefevre et al., 1995; Malviya et al., 1990; Pesty et al., 1998; Smith and Wells, 1983a; Smith and Wells, 1983b; Smith and Wells, 1984]. For example, HeLa cells responded to the agonists ATP and histamine with a Ca^{2+} transient in both the cytosolic and nuclear compartments. Cytosolic Ca^{2+} concentrations were consistently greater than that of nuclear Ca^{2+} concentrations. The agonists effected this mobilization of intracellular Ca^{2+} via the generation of IP_3. The disparate magnitudes between nuclear and cytosolic Ca^{2+} concentration might have been attributed to local generation of IP_3-dependent Ca^{2+} signals. Several studies have shown that the nucleus possesses all the necessary components to independently control an inositol phosphate lipid cycle.

3.3.2. Nuclear Pore Complex (NPC)

The NPC is an immense heteromeric supramolecular biological unit that generally consists of at least 100 proteins with an approximate mass of 124 MDa [Pante and Aebi, 1996], and exhibits an aqueous aperture of 10 nm [Maul, 1977; Miller et al., 1991]. The NPC is responsible for the energy-dependant gating and bi-directional trafficking of macromolecules between the cytoplasmic and nucleoplasmic space. Studies have shown, using either electron or atomic force microscopy, that elevated Ca^{2+} or ATP levels can effectively block or plug the central pore. Furthermore, the NPC may also possess ionic channel characteristics. The NPC may be involved in Ca^{2+} signalling. This presupposes that the nucleus is readily exposed to large fluctuations of ionic movement throughout the cell. Several studies contradict this notion by demonstrating that the nucleus can be shielded from Ca^{2+} fluctuations and that the NPC apparently possesses a channel-like activity, which can be modulated [Bustamante et al., 2000; Mazzanti et al., 2001; Shahin et al., 2001].

The contention that the nucleus is shielded from large fluctuations in cytosolic Ca^{2+} concentrations [al Mohanna et al., 1994], may be indicative of the nuclear pore complex exhibiting a selectivity for Ca^{2+} [Bustamante et al., 2000]. Furthermore, the control of Ca^{2+} flux through the nuclear pore complex might be exerted via perinuclear luminal depletion. This is observed in cases where nuclear protein transport appears to be inhibited under conditions known to deplete perinuclear luminal Ca^{2+} stores [Bustamante et al., 2000; Lee et al., 1998; Pante and Aebi, 1996; Perez Terzic et al., 1997a; Stehno Bittel et al., 1995b; Stoffler et al., 1999]. The inherent pore size of the nuclear pore complex

has invariably fostered the assumption that Ca^{2+} ion permeability was very high. However, it has been shown that the nuclear envelope possesses a high electrical resistance [Gerasimenko et al., 1995; Maruyama et al., 1995], which eventually led to the demonstration that the nuclear pore complex has a discernable ion channel activity and may be regulated by ATP and other cytosolic constituents [Bustamante et al., 2000]. Many genetic processes in the nucleus are apparently Ca^{2+}-dependant. Therefore, it would be advantageous to regulate Ca^{2+} movements in the nucleus to avoid unregulated genetic transcription.

4. Nuclear/Cytosolic Ca^{2+} Signalling

Ca^{2+} is required throughout the life cycle of the cell, from inception, development, and ultimately death. Research has shown that Ca^{2+} oscillations are more effective at inducing gene expression than a comparable sustained increase in Ca^{2+} concentration [Dolmetsch et al., 1998; Li et al., 1998]. As a consequence of the nuclear membrane insulating the nucleus from large cytosolic Ca^{2+} fluctuations, a nucleocytoplasmic gradient is formed. The detection of this gradient is accomplished using fluorescent dyes. Although this technique is very sensitive and a valuable tool in Ca^{2+}-based research, it is heavily criticized for several reasons [Perez Terzic et al., 1997b]. First, the inherent characteristic of dyes allows them under certain conditions to act as buffers and misrepresent Ca^{2+} concentrations. Second, the dyes can compartmentalize in different subcellular organelles, and third, the dye may have differential responses dependent upon the subcellular environment. All of these problems results in inaccurate calibration of the dye. In an attempt to alleviate

the controversy concerning de-esterification depdendent loading of fluorescent dyes, techniques such as fluorescent reporter genes, and microinjection have been employed. These techniques are not susceptible to subcompartmentalization, but are still hampered by criticism of proper calibration and accuracy of these techniques.

The ability of the nuclear envelope to independently regulate nucleo/cytoplasmic Ca^{2+} gradients may result from its structural characteristics. One of these characteristics is the contiguous luminal nature shared between the nuclear envelope and the endo/sarcoplasmic reticulum. This theoretically allows the nuclear envelope access to an even larger pool of releasable Ca^{2+}. With various components such as, IP_3, IP_4, cADPr, and SERCA pumps localized to either the inner or outer membranes of the nuclear envelope, a model emerges by which Ca^{2+} signals could be translated into the nucleus.

The first studies that examined the effect of Ca^{2+} upon gene transcription was work on *c-fos* [Greenberg et al., 1986]. Subsequently, research has been able to demonstrate that Ca^{2+} fluctuations, either from the plasma membrane or intracellular stores, are capable of activating the expression of this gene [Schonthal et al., 1991]. Ca^{2+} also appeared to influence gene expression of the cyclic AMP response element binding protein (CREB) [Sheng et al., 1990]. Specifically, nuclear Ca^{2+} increases caused the recruitment of a co-activator, CREB binding protein (CBP) [Chawla et al., 1998; Jensen et al., 1991]. This in turn induces the activation of transcription by CREB, via binding to cyclic AMP

response element (CRE) and Ca^{2+}-response element (CARE) [Hai et al., 1989; Sheng et al., 1990].

The majority of the studies to date show Ca^{2+} has an indirect effect upon DNA transcription. Recently, studies have demonstrated that Ca^{2+} may have a direct effect upon transcription, without the involvement of an intermediary. The identity of this DNA-binding transcriptional regulator is an antagonistic modulator called, downstream regulatory element-antagonist modulator (DREAM) [Carrion et al., 1999]. DREAM is a Ca^{2+}-binding protein of the EF-hand classification [Craig et al., 2002]. It possesses four Ca^{2+}-binding domains, that upon stimulation by Ca^{2+} prevents a tetrameric DREAM from binding to downstream regulatory element (DRE) domains; thereby, repressing the expression of the prodynorphin gene [Carrion et al., 1998]. Currently, studies have implicated DREAM being involved in Alzheimer's [Buxbaum et al., 1998; Leissring et al., 2000] and apoptosis [Jo et al., 2001].

The field of nuclear Ca^{2+} research is still in its infancy. The uncertainty regarding the capacity of the nucleus to autonomously regulate Ca^{2+} fluctuations, along with a greater understanding of global cellular Ca^{2+} fluctuations, will be a focus for future study. With advances in equipment, chemicals, and techniques that increase the spatial and temporal resolutions with which we can study samples at a molecular or cellular level, it is inevitable that these perplexing questions about nuclear Ca^{2+} signalling and its implications will be resolved.

Experimental Procedures

Preparation of embryonic ventricular cardiomyocytes

Single cell suspensions of embryonic ventricular cardiomyocytes from 10 day-old embryonic chick hearts were enzymatically isolated by methods described by Bkaily et al [Bkaily et al., 1996]. Briefly, after chick embryos were aseptically removed from the egg (Fig. 1;A-D), the heart was immediately removed (Fig. 1;E), the atria and great vessels dissected away (Fig. 1;F), washed in SMEM containing 50 IU/ml penicillin G potassium (solution A), minced, and enzymatically dispersed using a gentle cyclical trypsin treatment. The trypsinization was performed at 24 °C using solution A containing 0.1 % trypsin for 5 min in a stirrer flask agitating slowly. The cell suspension was then discarded and the ventricular tissue was further exposed to another cycle of trypsinization. The cyclical enzymatic dispersion was repeated 3 times, where only the last trypsin cycle was retained. The cell suspension was then centrifuged at 1000 x ***g*** for 10 min at 4 °C and then resuspended in HMEM supplemented with 5 % FBS and 50 IU/ml penicillin G potassium (solution B). The isolated embryonic cardiomyocytes were plated on sterile 25 mm glass coverslips. Cultured embryonic ventricular cardiomyocytes were maintained up to 48 hr at 37 °C in solution B in 5 % CO_2 AND 95 % O_2.

Localization of SERCA2 and RyR2 in embryonic cardiomyocytes

Briefly, embryonic cardiomyocytes were cultured for 1 day on glass coverslips and then fixed in either 1 % paraformaldehyde and then permeabilized

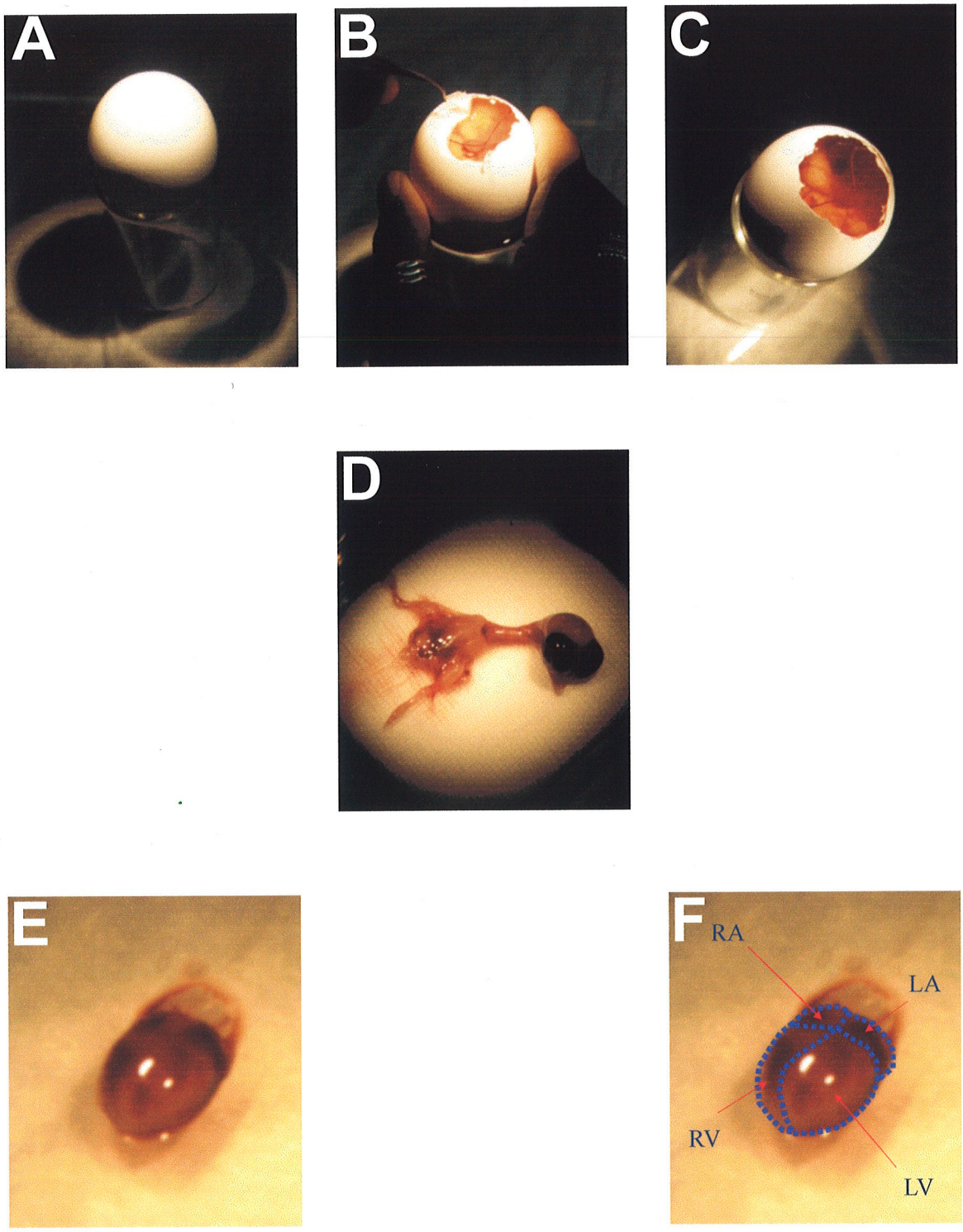

Figure 1. Embryonic heart isolation.
10-12 day old embryonic chick ventricular cardiomyocytes were isolated and cultured as described in 'Material and Methods'. Panels A-C demonstrate the procedure for exposing the embryo. Panel D shows the embryo after it has been removed from the egg and placed upon sterile gauze. After the removal of the heart from the embryo (panel E), the right/left atria (RA/LA) are separated from the right/left ventricles (RV/LV) as shown in the annotated image (panel F).

with 0.1 % Triton-X 100 for immunocytochemistry or live, unfixed for probing with BODIPY fluorescent derivatives of thapsigargin and ryanodine. Fixed cells were then adequately washed in PBS. Immunocytochemistry of fixed cells followed standard procedures, where separate slides were incubated with either a primary antibody against SERCA2 or RyR2 for 1 hr at a dilution of 1:1000. Resolving primary antibody staining was achieved by incubation of the cells with an Alexa secondary antibody at a dilution of 1:5000, for 1 hr. Probing of fixed cells with the BODIPY fluorescently conjugated forms of thapsigargin and ryanodine were performed by incubating the cells in the dark for 1 hr with concentration of 1 μM and 100 nM, respectively. All experiments were performed at room temperature. Cells were subsequently imaged using confocal microscopy.

Confocal imaging of Ca^{2+} fluorescence

Embryonic cardiomyocytes cultured for 1 day were washed in Tyrode's buffer. Cells were then loaded with 13 μM Fluo-3, AM, a Ca^{2+}-sensitive membrane permeant fluorescent dye, in Tyrode's supplemented with 0.1 % BSA for 45 min. Cells were then washed in Tyrode's for another 15 min to allow for complete de-esterification of dye. Immediately before visualization cells were briefly washed with an intracellular buffer (20 mM NaCl, 130 mM KCl, 2 mM $MgCl_2$, 5 mM HEPES, pH 7.2) and then replaced with intracellular buffer containing 100 nM $CaCl_2$. Images of Ca^{2+} fluorescence were acquired using a BioRad MRC-600 confocal imaging system. Cells were then monitored and exposed to a transient 5 μM ionomycin-perforation, which was promptly removed immediately after a response to ionomycin was observed. Cells were then

washed with intracellular buffer and a series of z-sections were obtained at each extranuclear bolus addition of Ca^{2+} from 100 to 1600 nM. At the conclusion of the experiment, nuclei were stained with 200 nM Syto-11 for 10 min. All experiments were performed at 27 °C.

Cellular Viability Assay

Embryonic cardiomyocytes were cultured for 1 day and gently washed with PBS. Cells were then exposed to a short, 30 s and prolonged, 10 min treatment to 5 μM ionomycin. Immediately after ionomycin-perforation, cells were loaded with optimized concentrations of LIVE/DEAD® assay reagents (Molecular Probes, Inc.): 0.1 μM Calcein, AM and 2.5 μM EthD-1, for 45 min in the dark at room temperature. Following incubation with LIVE/DEAD® assay reagents, cells were imaged using a confocal microscope.

Image and Statistical Analysis

Image and volume rendering was performed using ImageSpace software for SGI and statistical analysis using Graphpad Prism v.3.1 software for PC. All figures represented by bar charts of nuclear Ca^{2+} is based upon the volumetric mean Ca^{2+} fluorescence intensity attributed to either the nucleus or the cytosol where indicated.

Results

SERCA2 and RyR2 localization

Figure 2 shows confocal images of SERCA2 and RyR2 localized in 1 day cultured 10 day-old embryonic ventricular cardiomyocytes as described in the Material and Methods (Fig. 1). Localization was performed either by immunocytochemistry or BODIPY fluorescently conjugated ryanodine and thapsigargin for RyR2 and SERCA2, respectively. The images show relatively high RyR2 and SERCA2 staining intensity around the nucleus in regions referable to sarco/endoplasmic reticulum and nuclear envelope regions of the cell [Bkaily et al., 1996]. This is consistent with previous localization studies [Downie et al., 1998; Gerasimenko et al., 1995; Lanini et al., 1992]. The intense co-localization of RyR2 and SERCA2 in this region of embryonic chick ventricular cardiomyocytes suggests they may be involved in regulating nucleoplasmic Ca^{2+}.

Nuclear Ca^{2+} loading of embryonic cardiomyocytes

To examine how RyR2 and SERCA2 were involved in this process we first studied the spatial distributions of Ca^{2+} loading in ionomycin-perforated cells. Using the fluorescent dye Fluo-3 to monitor Ca^{2+} dynamics, a relatively homogenous distribution of fluorescence throughout the cell is ordinarily observed. Under conditions that cause Ca^{2+} fluctuations, extranuclear increases in fluorescence intensity are observed that can be attributed to either the cytoplasm or reticular membrane structures. Our interest in the role of nuclear envelopes in regulating nucleoplasmic Ca^{2+} arises from the common observation in quiescent, unperforated embryonic chick ventricular cardiomyocytes of a halo

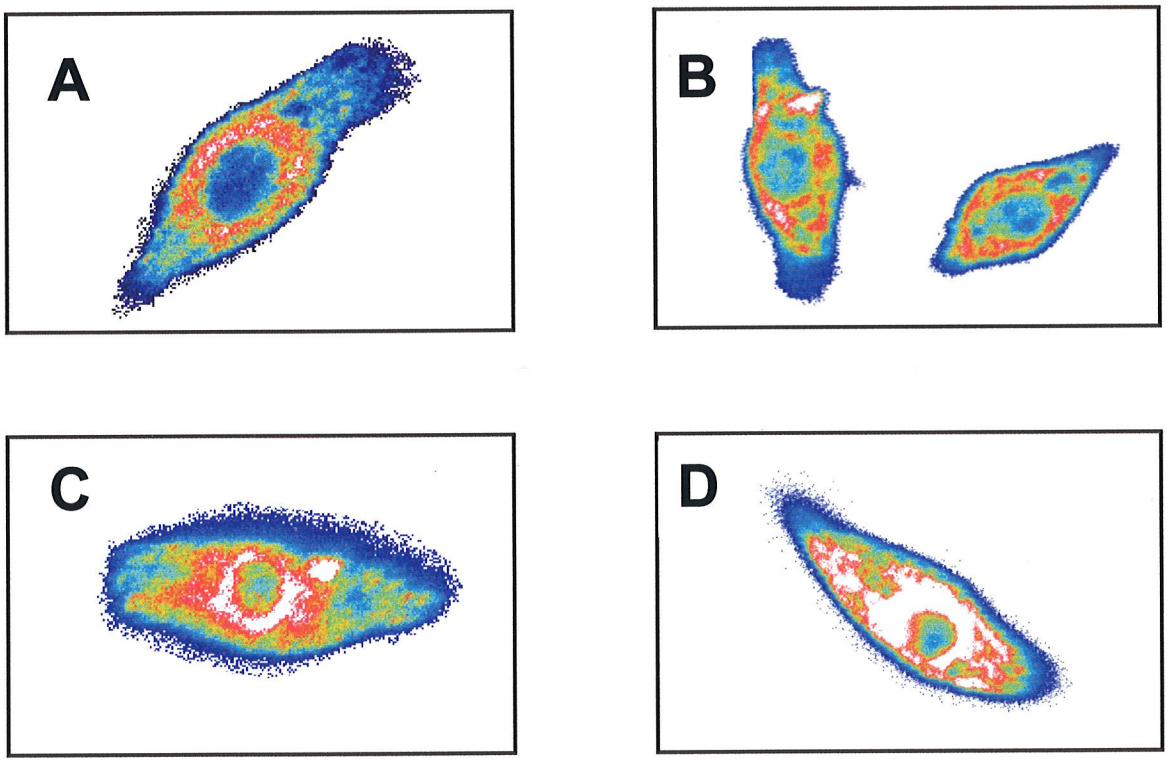

Figure 2. Immunocytochemical localization of SERCA2 and RyR2 to the nuclear envelope.

In panels A and B, isolated embryonic cardiomyocytes were probed with antibodies to SERCA2 and RyR2, respectively. In panels C and D, embryonic cardiomyocytes were treated with the fluorescent BODIPY FL derivatives of Tg and ryanodine, respectively. These results have shown that both immunocytochemical techniques highly localize SERCA2 and RyR2 proteins to a region referable as the nuclear envelope.

of Fluo-3 fluorescence intensity referable to the perinuclear luminal space (Fig. 3;Ai). Occasionally, this intense halo staining would progressively increase followed by a spontaneous nucleoplasmic flash (Fig. 3;Aii,iii). This suggests that the perinuclear halo may be a source of nucleoplasmic Ca^{2+}. Panel B shows representative z-section images of these embryonic cardiomyocytes with increasing extranuclear Ca^{2+} concentrations. The majority of the fluorescence increase was attributable to the nucleus and was readily discernible at 200 nM extranuclear added Ca^{2+}. The Syto-11 staining (as described in the Material and Methods) was used to effectively segregate the nuclear Ca^{2+} fluorescence from that of the cytosolic Ca^{2+} fluorescence. Panel C shows the results of separating the volumetric mean Ca^{2+} fluorescence intensity attributable to either the cytosol or the nucleus. As the added extranuclear Ca^{2+} concentration increased a subsequent increase was observed in the nucleus, with a significantly smaller fluorescence increase observed in the cytosol. Interestingly, nucleoplasmic signals could not be washed out following loading. This suggests Ca^{2+} is not freely permeable between the cytosol and nucleoplasm.

Effect of ionomycin-perforation on nuclear Ca^{2+} loading and cellular viability

The success of this technique relies heavily on perforation of only the sarcolemma. The reticular Ca^{2+}-dependent Fluo-3 staining patterns appear consistent with preserved functional integrity of intracellular membranes. Figure 4 shows the volumetric mean nuclear Ca^{2+} fluorescence of a) transiently perforated, b) unperforated, and c) overperforated embryonic cardiomyocytes with 5 μM ionomycin. As shown, nucleoplasmic Ca^{2+} loading relies on

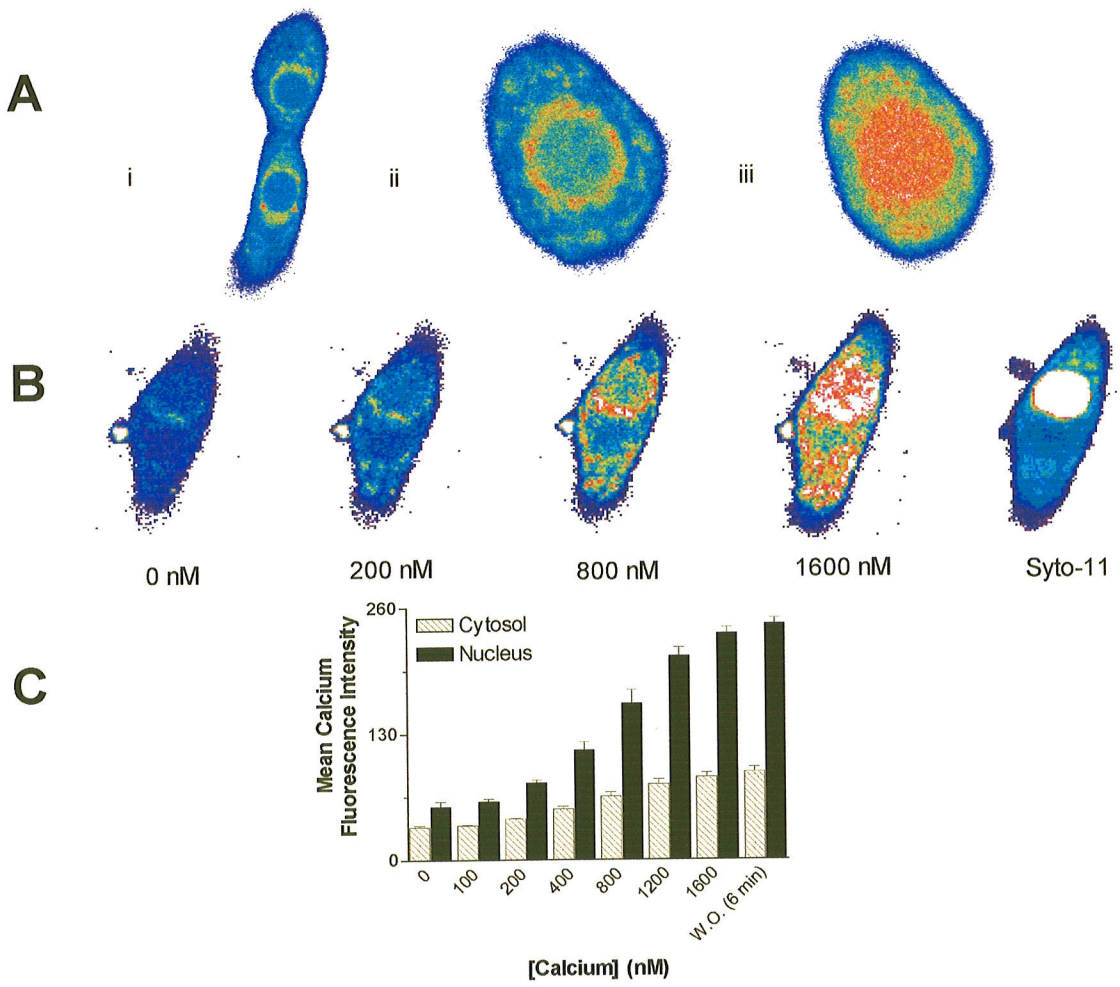

Figure 3. Nuclear Ca^{2+} transport in ionomycin-perforated embryonic cardiomyocytes.

Isolated embryonic cardiomyocytes were loaded with 13 μM Fluo-3, AM and a series of Z-sections were obtained using confocal microscopy as described in the Material and Methods. In panel A, confocal images of embryonic cardiomyocytes resolved an annular ring of Ca^{2+}-dependent fluorescence, referable to the perinuclear luminal space. An apparent spontaneous mobilization of this Ca^{2+} pool into the nucleoplasm was observed. Panel B shows representative z sections of cardiomyocytes that were transiently perforated with 5 μM ionomycin and increasing concentrations of Ca^{2+} (0–1600 nM) were added to the bathing buffer every 2 min. Cardiomyocytes were then treated with 200 nM Syto-11 in order to visualize and segregate the nucleus. In panel C, confocal images were then analyzed to obtain both cytosolic and nuclear Ca^{2+} fluorescence. These observations indicated that increases in added Ca^{2+} concentration caused a mean nuclear Ca^{2+} fluorescence intensity increase with relatively very little change in cytosolic fluorescence.

membrane integrity and depends on cytosolic Ca^{2+} concentration. Intact and overperforated cells showed no increases in nucleoplasmic Ca^{2+} with increasing bath Ca^{2+}. Strong nucleoplasmic signals were only successfully recorded in transiently perforated cells. This substantiates the assumption that: a) transient ionomycin-perforation was sufficient to allow for the free passage of Ca^{2+} ions from the extracellular bathing buffer into the cytoplasm and subsequently into the nucleoplasm, b) the increase in nuclear Ca^{2+} fluorescence was not caused by a direct influx through L- or T-type channels, Na^{+}/Ca^{2+} exchanger, or activation of several other Ca^{2+}-dependent pathways such as Ca^{2+}-induced Ca^{2+} release, and c) the transient ionomycin-perforation was not overly aggressive as to potentially alter cellular dye retention and nuclear membrane integrity. The transient ionomycin-perforation is a suitable model for investigating the role of the nuclear envelope in actively mobilizing Ca^{2+} from the cytoplasm into the nucleus.

To investigate preservation of nuclear integrity following ionomycin-perforation, we performed a LIVE/DEAD® viability assay. Figure 5 shows the time-dependent effect of ionomycin-perforation upon embryonic cardiomyocyte viability. In panels A and B, viable, Calcein green staining in unperforated and transiently perforated embryonic cardiomyocytes is shown. Conversely, panel C shows non-viable, EthD-1 red fluorescent nuclei after a 10 min incubation with ionomycin. Furthermore, prolonged exposure to ionomycin caused dramatic alterations in cellular morphology characterized by a loss of an ovoid appearance (rounded) and loss of de-esterified dye retention (data not shown). Thus,

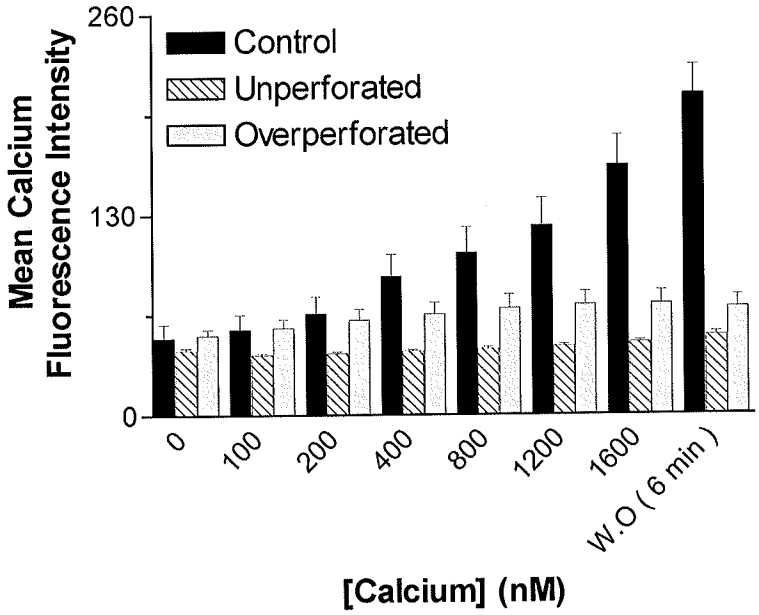

Figure 4. Ionomycin-perforated dependence upon active nuclear Ca^{2+}-uptake.

Fluo-3, AM loaded embryonic cardiomyocytes in the absence or presence of a short or prolonged 5 μM ionomycinperforation were visualized as increasing Ca^{2+} concentrations (0–1600 nM) were added to the bathing buffer. These experiments confirmed that transient ionomycin perforation sufficiently allowed free passage of added Ca^{2+} from the bathing buffer into the cell, but was not the causal factor in the uptake of Ca^{2+} into the nucleus and did not cause detrimental damage to the cell as compared to that of the prolonged, overperforated condition.

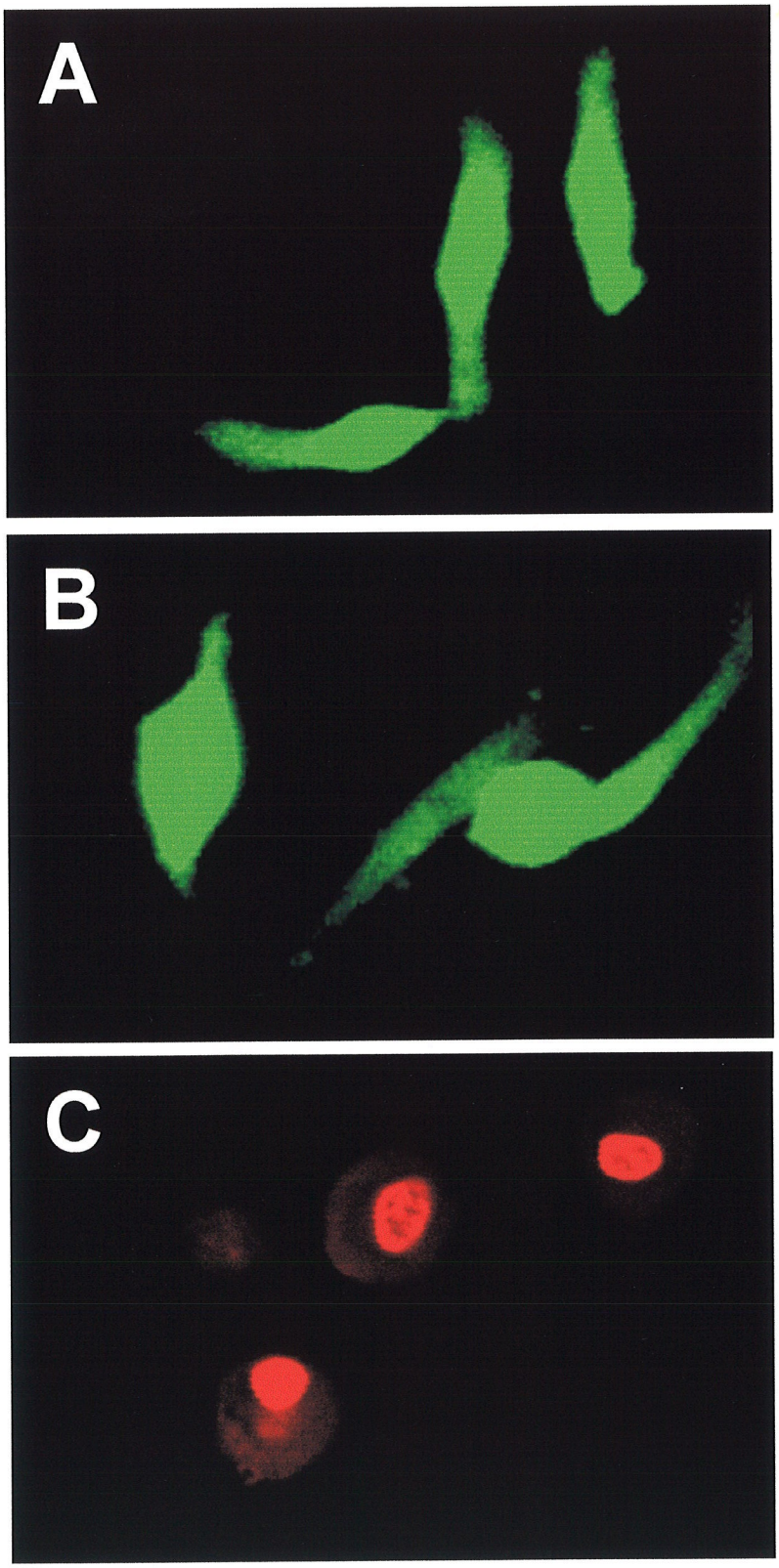

Figure 5. Effect of ionomycin-perforation duration upon embryonic cardiomyocyte viability.

Isolated cardiomyocytes were prepared and loaded with LIVE/DEAD® assay reagents, as described in the Material and Methods. In panel A, isolated embryonic cardiomyocytes were untreated and not exposed to ionomycin. In panels B and C, isolated embryonic cardiomyocytes were subjected to a short 30 s and prolonged 10 min exposure to 5 μM ionomycin, respectively. These results indicated that the short exposure to ionomycin did not affect the viability of the cell as compared to controls, while prolonged exposure caused dramatic morophological cellular changes.

transient ionomycin-perforation appears not to structurally or functionally impair intracellular membrane systems.

Effects of thapsigargin, CPA, or ryanodine on nuclear Ca^{2+} loading

To assess the role of SERCA2 pumps in nucleoplasmic loading, we examined effects of SERCA-specific inhibitors thapsigargin and CPA upon nuclear Ca^{2+} loading (Fig. 6). A concentration of 5 μM thapsigargin was usually sufficient to maximally inhibit nuclear Ca^{2+} loading. Low (0.1 μM) thapsigargin concentrations produced negligible effects upon inhibiting nucleoplasmic Ca^{2+} loading (data not shown). Slightly less potent inhibition with 10 μM CPA was observed. In our hands, higher inhibitor concentrations (> 20 μM) caused detachment and loss of specimen. The inhibitor concentrations employed here did not discernibly alter cellular morphology. The implications of this experiment is that SERCA2 pumps localized to the nuclear envelope (Figure 2, panels A and C) are functionally active in embryonic ventricular cardiomyocytes. This is in accordance with other previous studies that have shown that the SERCA pump is directly involved in regulating the movement of Ca^{2+} fluxes at the level of the nuclear envelope [Gerasimenko et al., 1995].

We then performed complimentary experiments examining the concentration dependent effects of ryanodine (Fig. 7). At low (0.1 μM) ryanodine concentrations, the cytoplasmic Ca^{2+} sensitivity of nucleoplasmic Ca^{2+} loading was increased. These concentrations increased the open probability of single RyR2 channels [Buck et al., 1992]. However, at ryanodine concentrations

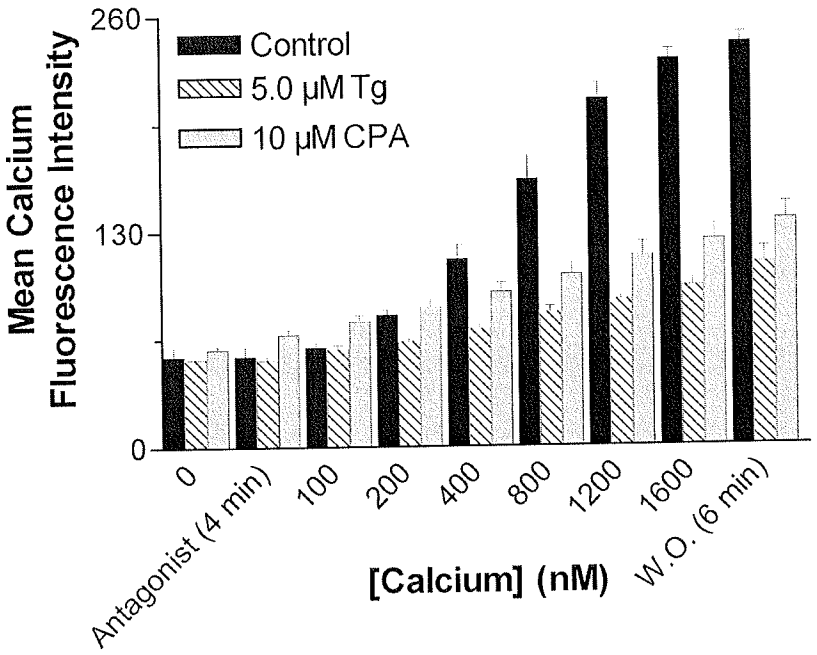

Figure 6. Tg and CPA inhibition of nuclear Ca^{2+} transport in ionomycin-perforated embryonic cardiomyocytes.

Isolated cardiomyocytes were prepared and loaded with 13 µM Fluo-3, AM as described in 'Material and Methods'. Both Tg and CPA were added after cells were transiently perforated with 5 µM ionomycin. These observations indicated both SERCA pump inhibitors abrogated nuclear Ca2+-uptake into nuclei, as compared to controls. Furthermore, inhibition by Tg was concentration dependent.

(10 μM) producing subconducting, apparently "inhibited" channels, nucleoplasmic loading was markedly reduced [Rousseau et al., 1987]. These effects of ryanodine suggests RyR2 activation opens a pathway between cytosolic and nucleoplasmic Ca^{2+} pools. Inactivating the pathway then blocks the passage of Ca^{2+} from the cytosol to the nucleoplasm. These results support the finding of RyR2 at the nuclear envelope (Figure 2, panels B and D) in embryonic chick ventricular cardiomyocytes. This is further substantiated by the localization of RyR to the nuclear envelope in other cell types [Gerasimenko et al., 1995; Santella and Kyozuka, 1997]. Hence, the RyR2 localized in these cells are in fact functional and responsive in accordance with other ryanodine studies involving vesicular or patch clamp studies [Holmberg and Williams, 1990; Meissner, 1986].

Why ryanodine produces these bimodal effects on RyR2 and whether their observance here speaks to the arrangement of channels in nuclear envelopes is not known. Upon further investigation, we found that co-administration of inhibitory thapsigargin and ryanodine concentrations produced an unexpected supramaximal increase in nucleoplasmic Ca^{2+} fluorescence (Fig. 8). A slightly less potent response was similarly observed with 10 μM CPA. Interestingly, supramaximal increases were still produced in the presence of inhibitory concentrations of thapsigargin and activatory concentrations of ryanodine. This effect was independent of the order of addition and suggests complex involvement of SERCA2 and RyR2 in nucleoplasmic Ca^{2+} regulation.

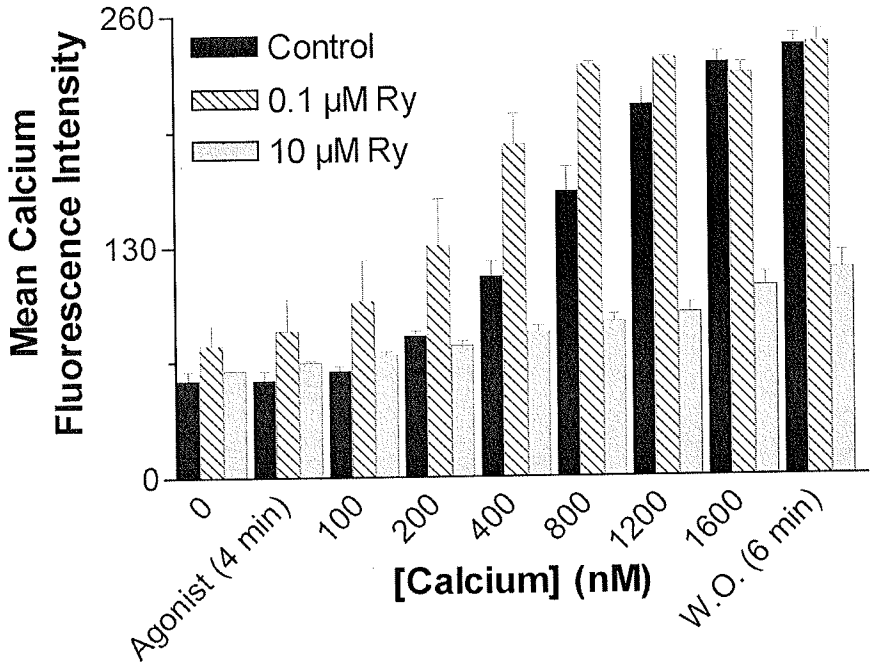

Figure 7. Ryanodine inhibition of nuclear Ca^{2+} transport in ionomycin perforated embryonic cardiomyocytes.

Isolated cardiomyocytes were prepared and loaded with 13 µM Fluo-3, AM as described in 'Material and Methods'. Ryanodine was added after cells were transiently perforated with 5 µM ionomycin. These observations show a bimodal ryanodine effect upon nuclear Ca^{2+}-uptake. Low ryanodine concentrations cause an activation of nuclear Ca^{2+} transport, while higher concentrations inhibit the active mobilization of Ca^{2+} into the nucleoplasm.

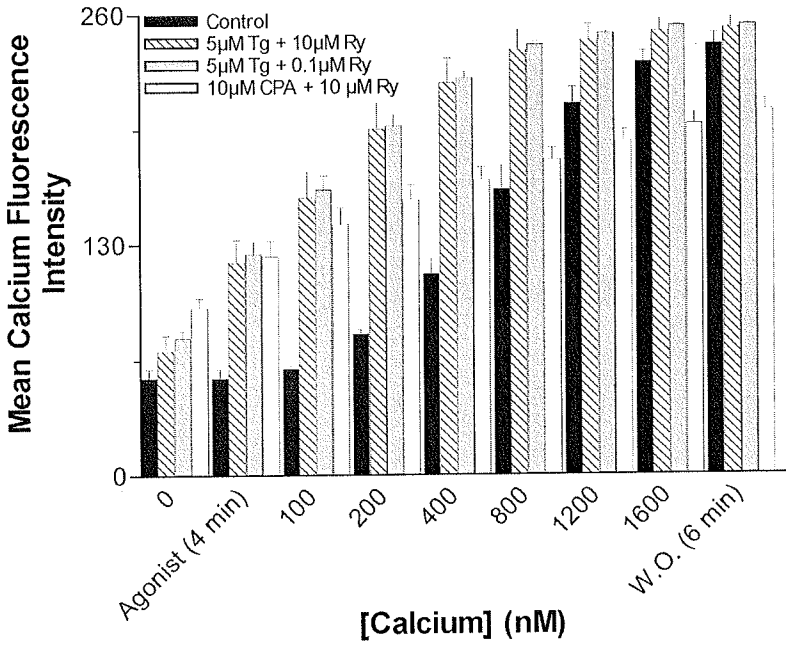

Figure 8. Effect of concomitant Tg with Ry or CPA with Ry addition upon nuclear Ca^{2+}-uptake.

Isolated cardiomyocytes were prepared and loaded with 13 μM Fluo-3, AM as described in the Material and Methods. 5 μM Tg or 10 μM CPA with either 0.1 μM or 10 μM Ry were added after cells were transiently perforated with 5 μM ionomycin. These experiments show that the combined addition of Tg or CPA with Ry appear to cause a further activation of nuclear Ca^{2+}-uptake as compared to controls.

Discussion

In this study we investigated whether nucleoplasmic Ca^{2+} can be independently regulated by Ca^{2+} exchanges across nuclear envelopes in embryonic cardiomyocytes. Our studies demonstrate a discrete localization of SERCA2 and RyR2 proteins to a perinuclear halo region (Fig. 2). Both proteins were found to be play important roles in nucleoplasmic Ca2+ regulation (Fig. 6-8). Our observations indicate nuclear envelopes play an active role as a transduction/limited diffusion domain for cytosolic and nucleoplasmic Ca^{2+} exchanges.

The underlying mechanism regulating nucleoplasmic Ca^{2+} concentrations is not clear and opinions over this are quite diverse [Gerasimenko et al., 1996; Lanini et al., 1992; Perez Terzic et al., 1997a; Stehno Bittel et al., 1995b]. One question is whether (a) the nuclear pore is freely permeable to Ca^{2+} and is the primary pathway for nuclear Ca^{2+} entry or (b) whether transport proteins in perinuclear regions can contribute to nucleoplasmic Ca^{2+} regulation. Perhaps both processes operate in conjunction with each other. Earlier confocal imaging studies [al Mohanna et al., 1994] indicated nucleoplasmic Ca^{2+} was freely accessible to the cytosol at low cation concentrations. However, at high cytosolic Ca^{2+} the nucleoplasm appeared shielded from further Ca^{2+}-dependent fluorescence increases [al Mohanna et al., 1994; Bkaily et al., 1996]. Several other reports have shown cytosolic Ca^{2+} wave propagation into the nucleoplasm [Bkaily et al., 1996; Genka et al., 1999; Ikeda et al., 1996; Lin et al., 1994]. The degree to which these are temporally separated and, therefore, attributed to one

or other transport process is still debated [Minamikawa et al., 1995]. Nevertheless, there is considerable immunological and functional evidence implicating nuclear envelope Ca^{2+} transport proteins (e.g. SERCA, RyR, IP_3 and IP_4 receptors, R-type Ca^{2+} channels) in mobilizing Ca^{2+} between perinuclear cisterns and the nucleoplasm [Berridge, 1993; Bkaily et al., 1997; Divecha et al., 1993; Gerasimenko et al., 1995; Hagar et al., 1998; Koppler et al., 1993; Malviya et al., 1990; Pesty et al., 1998; Santella and Kyozuka, 1997].

In interpreting the functional effects of thapsigargin and ryanodine found in this study it seems imperative to consider their potential effects upon Ca^{2+} permeability through nuclear pore complexes. Perinuclear Ca^{2+} store depletion by thapsigargin reveals complex structural and functional alteration of nuclear pores involving migration of an occluding central plug and inhibition of macromolecular transport [Lee et al., 1998; Perez Terzic et al., 1997a; Perez-Terzic et al., 1996; Stehno Bittel et al., 1995b; Wang and Clapham, 1999]. What this does to pore-mediated Ca^{2+} ion conductance is not known because Ca^{2+} permeation characteristics of nuclear pores have not been determined. Thapsigargin might conceivably prevent Ca^{2+} entry through apparently inhibited nuclear pores complexes. However, active filling of perinuclear Ca^{2+} cisterns promotes macromolecular transport, which has been recently shown to also attenuate large ion conductance (g) activity through nuclear pores [Bustamante et al., 2000]. Thus, it is not known to what extent and under what conditions nuclear pores permit free Ca^{2+} ion passage. On the other hand, thapsigargin commonly produces transient nucleoplasmic Ca^{2+} flashes in non-perforated

embryonic cardiomyocytes and isolated nuclei (Gilchrist, Massaeli, Czubryt, Abrenica, unpublished observations). These flashes slowly fade and their observation is consistent with thapsigargin acting to (a) first indirectly activate perinuclear Ca^{2+} release channels by SERCA inhibition [Dettbarn and Palade, 1998] and (b) then directly inhibit capacitative Ca^{2+} re-entry.

Whether thapsigargin inhibits SERCA pumps on both inner and outer nuclear membranes is not clear although functional and immunolocalization studies seem to support this idea [Downie et al., 1998; Lanini et al., 1992; Nicotera et al., 1989]. Whether and how SERCA pumps can account for spontaneous and sustained nucleoplasmic flashes apparently coordinated with progressive increases in perinuclear Fluo-3 signal intensity is also not clear (Fig. 3). These large flashes appeared in cells apparently committed to apoptosis with subsequent observation of surface blebbing. One possibility is that progressive increases in ER/SR and perinuclear fluorescence in these cells reflect imbalances in Ca^{2+} transport mechanisms coordinating capacitative Ca^{2+} filling of intracellular stores [Mogami et al., 1998]. The net result may be luminal Ca^{2+} activation of Ca^{2+} channels on inner nuclear envelope membranes. In all cells exhibiting these spontaneous phenomena nucleoplasmic signal intensity was sustained. This suggests absence of free Ca^{2+} permeation through nuclear pore complexes at this point. The possibility that nuclear pore complexes close at elevated perinuclear Ca^{2+} store levels may also account for the absence of Fluo-3 signal wash-out shown here following nuclear Ca^{2+} loading in perforated cardiomyocytes. Indeed, these observations are consistent with the idea that

filling of nuclear cisterns shuts down nuclear pore ion conductance [Bustamante et al., 2000] and shields nucleoplasmic compartments from the cytosol.

Although our data implicates SERCA pumps in nucleoplasmic Ca^{2+} regulation the thapsigargin effects observed here remain ambiguous with respect to identifying modes of nuclear Ca^{2+} entry. However, a clearer picture emerges from our observations with ryanodine. Whether ryanodine directly or indirectly affects nuclear pore structure is not known. At low ryanodine (0.1 μM) concentrations 80 % higher nucleoplasmic Fluo-3 signal intensity was recorded at sub-micromolar Ca^{2+} concentrations (Fig. 7). These conditions would be expected to promote RyR2 Ca^{2+} channel opening [Lai and Meissner, 1989; McGrew et al., 1989; Pessah et al., 1997; Rousseau et al., 1987; Zimanyi et al., 1992]. Since RyR channels permit bi-directional Ca^{2+} flux [Tripathy and Meissner, 1996] then signal increases might reflect opening of a discrete Ca^{2+} conduit across perinuclear cisterns. Whether this involves RyRs on inner, as well as, outer nuclear membranes is not known [Santella and Kyozuka, 1997]. However, we presume inner nuclear membrane Ca^{2+} permeability must have increased in response to ryanodine because nucleoplasmic signals were resistant to washout (Fig. 7). In addition, its identity as an independent pathway also seems likely given ryanodine-mediated signal increases were unaffected by inhibitory actions of thapsigargin (Fig. 8).

The existence of a discrete ryanodine-sensitive perinuclear Ca^{2+} pathway is also indicated by complete attenuation of nucleoplasmic signal increases produced at high ryanodine concentrations known to induce formation of a

inhibited sub-conducting RyR2 Ca^{2+} channels (Fig. 8). How ryanodine induces these inhibitory states is unclear. Their formation requires (a) specific assay conditions (prolonged incubation, elevated temperature and high luminal Ca^{2+}) and (b) initial ryanodine-mediated RyR activation [Gilchrist et al., 1992; Meissner, 1986; Pessah et al., 1997; Rousseau et al., 1987]. In other words, to induce inactivated RyR states channels must first be activated. However, a curious result was the supra-maximal activation of nucleoplasmic signal intensity when inhibitory concentrations of both thapsigargin and ryanodine were administered (Fig. 8). The most promising explanation for this result is that SERCA pumps may have a role in forming ryanodine inactivated states. We do not see how ryanodine-inactivated RyR2 states could reverse thapsigargin-induced effects. The basis for our proposal is that RyR Ca^{2+} channels appear sensitive to Ca^{2+} gradients [Tripathy and Meissner, 1996]. One hypothesis is that gradient control occurs through a luminal-to-cytosolic regulatory flux mechanism in RyR channels [Tripathy and Meissner, 1996]. Single channel studies have shown highly luminal-to-cytosolic Ca^{2+} fluxes inactivate RyR channels with opening observed at lower flux rates [Tripathy and Meissner, 1996]. *In situ*, this inactivated state may occur following SERCA-mediated luminal Ca^{2+} filling. Thus, collapsing luminal-to-cytosolic Ca^{2+} gradients by inhibiting SERCA pumps may prevent formation of inactive RyR states. This proposal clearly requires further investigation. However, it is a highly reproducible phenomenon and is similarly efficacious when cyclopiazonic acid was employed. What it does indicate is that SERCA pumps and RyR channels, distributed in highly co-localized perinuclear

regions, exert potentially complex influences upon nuclear Ca^{2+} regulation. The curious SERCA/RyR interaction observed here hints at a basis for understanding how Ca^{2+} waves may be propagated from the cytosol into the nucleoplasm. While the intracellular arrangement of SERCA pumps and RyR channels may serve the needs of developing embryonic nuclei, the broader significance of our findings is that there appears to be a complex interplay of SERCA and RyR function. This possibly influences signal activation and signal termination during Ca^{2+}-induced Ca^{2+} release and capacitative Ca^{2+} re-entry. Although cytosolic Ca^{2+} wave propagation is generally considered to involve Ca^{2+} release through IP3Rs [Clapham et al., 1993; Hagar et al., 1998; Stehno Bittel et al., 1995a], the presence of RyRs at perinuclear regions may reflect some role in triggering Ca^{2+} signals or involvement in regulating perinuclear and nucleoplasmic Ca^{2+} concentrations.

We have examined the role of the nuclear envelope in regulating the mobilization of Ca^{2+} in chick embryonic cardiomyocytes and have shown that the nuclear envelope possesses both SERCA2 and RyR2, thus allowing the nucleus to actively and independently regulate nuclear Ca^{2+} transport. This supports the evidence that the nuclear envelope serves as a storage site for Ca^{2+} and as an effective barrier to Ca^{2+} fluctuations within the cell [al Mohanna et al., 1994; Gerasimenko et al., 1995]. The presence of such a mechanism may transduce cytosolic Ca^{2+} oscillations via activation of Ca^{2+}-dependent transport processes within the nucleus. Our observations indicate that this occurs through a complex interaction between SERCA pumps and RyR channels.

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