Chapter 10

Communication in the Heart: Cardiokines as Mediators of a Molecular Social Network

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CARDIOKINES AND THE MOLECULAR SOCIAL NETWORK OF CELL COMMUNICATION

Communication between individuals of a society is essential for effective functioning of the whole. Tissues, organs, and organisms are, in essence, societies of cells that must communicate over short and long distances. Communication between cells over short distances relies upon physical association, whereas communication over long distance depends upon secretion, which is the process by which substances are transferred across an intact plasma membrane from intracellular to extracellular spaces. The heart is comprised of numerous cell types that communicate by secretion with each other, and with cells in other tissues. Although the chemical nature of the molecules secreted by the heart is varied, this review focuses mainly on proteins secreted by the heart, or cardiokines (1). Cardiokines and their receptors constitute a molecular social network that is organized to optimize interactions between cells in the heart, as well as communication between the heart and other tissues. As critical elements of this network, cardiokines contribute in a combinatorial fashion to the intricate communication system that forms the basis of acute and chronic responses of the cells in the heart to environmental cues.

In the first section of this chapter we review the archetype cardiokine, ANP, the first element of the molecular social network in the heart to be identified. We have used ANP as an example to describe the varied mechanisms by which cells synthesize and release cardiokines, as well as to highlight novel features of the molecular social network of the heart that distinguish it from most other secretory tissues. In the second section of the chapter, we examine a few of the more recently described elements of the network that illustrate the complexities, as well as the subtleties of communication in, and from the heart.

MECHANISMS OF SECRETORY PROTEIN SYNTHESIS, PROCESSING, AND SECRETION

Cardiokines and the Molecular Social Network

The discovery in 1981 that the heart is an endocrine organ has led to many studies focused on determining the nature of the communication substances released from the heart, as well as the mechanisms that govern their synthesis and secretion. Although heart cells secrete various types of molecules, the bulk of them are proteins, or cardiokines. Since most cardiokines are relatively large and hydrophilic, crossing the hydrophobic plasma membrane without compromising its integrity constitutes a significant challenge. Cells have met this challenge by employing several routes of cardiokine secretion that can be generally classified as either classical or non-classical protein secretory pathways (1). Moreover, the heart is a particularly complex secretory organ, because in addition to performing its main function of pumping blood, the muscle cells in the different chambers exhibit different cardiokine expression and secretion properties. For example, atrial myocytes store cardiokines in large dense-core vesicles (LDCVs) from which release is regulated, whereas ventricular myocytes store cardiokines in small vesicles from which release is constitutive.

Classical and Non-Classical Secretory Pathways

The Classical Secretory Pathway (Figure 10.1, A–D)

The first well-characterized pathway of protein secretion, called the classical secretory pathway, involves the

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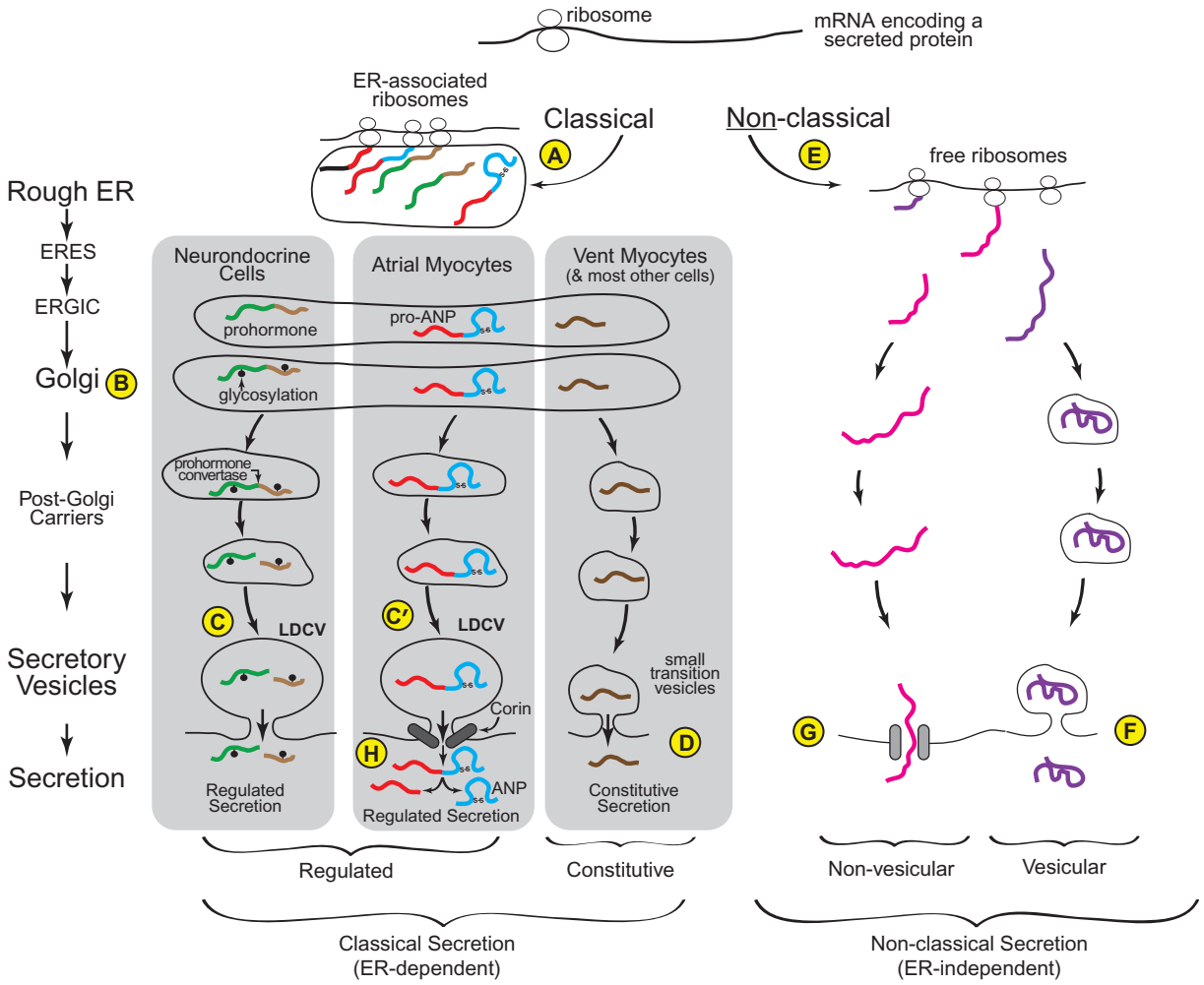

FIGURE 10.1 Mechanisms of cardiokine synthesis and secretion. Messenger RNAs encoding proteins destined for either the classical (A) or non-classical (E) secretory pathways are translated on either ER-associated, or free ribosomes, respectively. *Classical secretory pathway:* Cardiokines synthesized and secreted via the classical secretory pathway are transported from the ER lumen, to the Golgi (B), and eventually packaged in either large dense-core secretory vesicles (LDCVs) in cells such as endocrine cells, e.g. atrial myocytes, that exhibit regulated secretion, or packaged into small transition vesicles (D) in all cells, including neuroendocrine cells that exhibit forms of constitutive secretion. In neuroendocrine cells, some prohormones are glycosylated in the ER and Golgi lumen; almost all prohormones are subjected to post-translational proteolytic processing by prohormone convertases in the Golgi and secretory vesicles (C). Atrial myocytes are different than other neuroendocrine cells, in that the major secretory protein made in the heart is stored as the prohromone pro-ANP (C). Concurrent with secretion, pro-ANP is cleaved by corin to form the secreted, circulating bioactive form of the cardiokine, ANP (H). Ventricular myocytes differ from atrial myocytes in that they exhibit primarily constitutive secretion and little, if any, regulated secretion. However, ventricular myocytes in the pathologic heart are similar to atrial myocytes, in that they express pro-ANP, which is co-secretionally processed to the bioactive product, ANP, by corin. *Non-classical secretory pathway:* Cardiokines synthesized and secreted via the non-classical secretory pathway are either packaged into vesicles post-translationally and secreted via a vesicle-dependent mechanism (F), or they are transported across the plasma membrane through channels or pores (G).

endoplasmic reticulum (ER)-dependent synthesis and co-translational translocation of nascent proteins across the ER membrane (2). Although this pathway was originally delineated in cells derived from endocrine and exocrine glands, such as β-cells and acinar cells in the pancreas, further studies demonstrated the existence of the ER-dependent classical secretory pathway in all mammalian cell types examined, including atrial and ventricular myocytes. Proteins destined for secretion via the classical pathway are co-translationally translocated into the lumen of the ER (Figure 10.1, A). Once in the ER, secretory proteins proceed to ER exit sites (ERES) where they are incorporated into the membrane-coating coat protein II (COPII) vesicles that leave the ER and move the cargo to the ER-to-Golgi intermediate compartment (ERGIC), after which they are routed to the Golgi (Figure 10.1, B). Post-Golgi carriers

then transport secretory proteins to either large dense-core vesicles (LDCV) (Figure 10.1, C), where they can be stored until receipt of a stimulus for exocytosis, a process called regulated secretion (3), or they can be packaged into small vesicles that fuse with the plasma membrane in a manner that is independent of a stimulus, a process which leads to the unregulated, or constitutive secretion of soluble proteins (Figure 10.1, D) (4). In addition to being responsible for the secretion of proteins that are not targeted to the regulated secretory pathway, the constitutive pathway is also responsible for the delivery of membrane proteins to organelles or the cell surface.

The Non-classical Secretory Pathway (Figure 10.1, E–G)

Further investigation of the mechanisms of protein secretion led to the more recent discoveries of proteins that are released from cells in an ER-independent manner, called non-classical secretion (5). The first proteins shown to be secreted via the non-classical pathway were interleukin-1beta (IL-1β), galectin, and fibroblast growth factor. However, ensuing studies have identified at least 100 additional proteins that are released from cells via the non-classical secretory pathway. Proteins secreted via the non-classical pathway lack a conventional signal peptide, and are synthesized on free ribosomes (Figure 10.1, E). After their synthesis, non-classically secreted proteins can take several routes across the plasma membrane, which can be broadly classified as either vesicle-dependent or vesicle-independent (6,7). In the vesicle-dependent pathways, proteins are made on cytosolic-free ribosomes, then following their synthesis, they can be endocytosed into secretory lysosomes, transported into exosomes, or they can reside within microvesicles that are shed from the cell surface (Figure 10.1, F). The vesicle-independent pathways generally involve the direct translocation of cytosolic proteins across the plasma membrane, usually through protein-conducting channels or pores (Figure 10.1, G).

Biosynthesis of Secreted Proteins – Implications for the Heart

Although both the classical and non-classical pathways are responsible for cardiokine secretion, most studies carried out to date have been performed on cardiokines secreted via the classical pathway (1). Moreover, the cardiokine made in the largest quantities in the heart is atrial natriuretic peptide (ANP), which is secreted via the classical pathway. Accordingly, the following section is limited to a discussion of the classical secretory pathway, and ANP is used as an example of a cardiokine.

Signal Sequences

Approximately half of the proteins secreted via the classical pathway possess an N-terminal 20–26 amino acid hydrophobic signal sequence that targets the nascent polypeptide to the ER (8). Ribosomes engaged in translation of proteins with an N-terminal signal sequence bind to the ER via an ER-localized receptor that is part of the signal recognition particle, or SRP (9,10). Thus, signal sequences not only play crucial roles in localizing nascent polypeptides and their ribosomes and mRNAs to the ER, but they also facilitate the co-translational transfer across the ER membrane of proteins destined for the classical secretory pathway. One of the first modifications of most secretory proteins that have an N-terminal signal sequence is the signal peptidase-mediated, co-translational removal of the signal sequence in the ER membrane by regulated intramembrane proteolysis, or RIP (11). Following signal sequence removal, translation ensues, culminating with the complete transfer across the ER membrane of a precursor form of the secreted protein, often called the prohormone. Most prohormones undergo further post-translational modifications, such as glycosylation, disulfide bond formation, proteolytic processing, and C-terminal α-amidation before attaining mature, fully bioactive status.

Proteolytic Processing

The post-translational proteolytic maturation of prohormones usually involves subtilisin/kexin-like endoproteases, called prohormone convertases, the mature forms of which reside in secretory granules of the classical secretory pathway (12,13). The prohormone convertases, which are sometimes abbreviated as PCs, comprise a family of seven subtilisin/kexin-like endoproteases including furin, PC1/3, PC2, PC4, PACE4, PC5/6, and PC7 (12). Prohormone convertases perform a specialized function by cleaving prohormones on the C-terminal side of selected single, paired, or tetra basic amino acid residues. The products of prohormone convertase-mediated cleavage are often trimmed further by carboxypeptidases, such as carboxypeptidases E and D, which also reside within the classical secretory pathway (14). Prohormone convertases and carboxypeptidases are responsible for the processing of many proteins made in the rough-ER and then either secreted, or routed to the cell surface, including neuropeptides (e.g. enkephalin and dynorphin), peptide hormones (e.g. oxytocin and somatostatin), growth and differentiation factors (e.g. the bone

morphogenetic protein/transforming growth factor-β family), receptors (e.g. Notch and insulin receptor), enzymes (e.g. PCs and matrix metalloproteinases), adhesion molecules (e.g. a chain of integrins and collagens), blood coagulation factors (e.g. von Willebrand factor and factor IX), and plasma proteins (e.g. albumin and α_1-microglobulin). Prohormone convertases function in a combinatorial fashion to generate the appropriate collection of product peptides from prohormones, and therefore, they have important roles as regulators of cell−cell communication (12). Interestingly, cardiac myocytes are unusual in that they do not express any of the prohormone convertases that are usually involved in prohormone processing in other secretory tissues (2). This finding implies that post-translational proteolytic processing of prohormones in the heart is atypical (see below).

C-terminal Peptidyl α-amidation

Many peptide hormones secreted via the classical secretory pathway require C-terminal α-amidation for full biological activity (15). Peptidyl α-amidation involves the replacement of the hydroxyl group of the C-terminal amino acid of a protein with an amino group. The enzyme responsible for this modification, peptidylglycine α-amidating monooxygenase, or PAM, is found in the highest concentrations in cells with LDCVs, where it resides in the lumen, as well as the membrane; PAM C-terminally amidates peptides that have the appropriate consensus sequence. Interestingly, the cell type exhibiting the greatest concentration of PAM is the atrial myocyte, which, paradoxically, is not known to express any secreted proteins that are C-terminally amidated, implying potentially novel roles for PAM in the heart (see p.132).

Timing and Location of Prohormone Processing in the Heart

A common theme among cells that synthesize and secrete proteins via the classical secretory pathway is that all of the post-translational processing required for the generation of the final, bioactive product peptide takes place prior to secretion. Accordingly, the mature bioactive hormone is either stored within LDCVs in those cells that release hormone in a regulated manner, and/or it is packaged into small vesicles from which the bioactive protein is released constitutively, essentially as soon as it is made (2). Interestingly, unlike other tissues that synthesize and release proteins via the classical secretory pathway, cardiac myocytes do not store the mature, bioactive form of ANP, but instead store a prohormone form of ANP, implying novel mechanisms of prohormone processing in the heart (see below).

ANP, THE ARCHETYPE CARDIOKINE

The Endocrine Heart: Discovery of ANP

Atrial Granules and Natriuretic Peptides

Several studies in the 1950s and 1960s used electron microscopy to examine the ultrastructure of various tissues (16−18). These studies revealed the presence of LDCVs in atrial myocytes, but not in ventricular myocytes. Since the atrial LDCVs were similar to peptide hormone-containing vesicles in other tissues, the authors of these reports postulated that in addition to serving a critical contractile role, atrial myocytes might also serve an endocrine function. However, it was not until 1981 that the endocrine function of the heart was discovered. Using bioassays, it was shown that when injected into rats, atrial tissue extracts, but not ventricular tissue extracts, exerted strong natriuretic and diuretic effects, as well as hypotension (19). In 1983, the active circulating form of the substance responsible for these effects, ANP, was isolated and shown to be a 28 amino acid peptide that possessed a single disulfide bond that was required for activity (20,21). Soon thereafter, in 1988, two additional natriuretic peptides structurally related to ANP were discovered in porcine brain (22,23). The two new natriuretic peptide members were named B- and C-type natriuretic peptides, or BNP and CNP, which, like ANP, are encoded on separate genes. BNP was later found in relatively high levels in the cardiac ventricles, which are believed to be the source of circulating BNP (24), while CNP is not made in the heart (23). Like ANP, BNP is also made in atrial myocytes, albeit, in much lower quantities than ANP.

Physiological Roles for ANP

Soon after its discovery, a multitude of specific physiological roles for ANP were elucidated (21). For example, ANP was shown to decrease vascular smooth muscle tone and peripheral resistance, to increase glomerular filtration rate and to inhibit sodium reabsorption in the kidneys. In addition to these endocrine effects, ANP, which is upregulated in hypertrophic, ischemic, and failing hearts, also exerts autocrine and paracrine protective, anti-hypertrophic roles on cardiac myocytes in the diseased heart.

ANP signaling is mediated by its binding to membrane-associated guanylyl cyclase receptors, also called natriuretic peptide receptors, or NPRs. Although there are two main subtypes of this family of NPRs, NPR-A and NPR-B, ANP binds preferentially to the NPR-A, through which it mediates most of its physiological effects. Genetically modified mice have been used to study the effects of ANP, NPR-A, and NPR-B gain- and loss-of-function *in vivo*; these studies have shown that the ANP

and NPR system is not only critical for regulating blood pressure, salt excretion, and water excretion, but also for modulating ventricular growth (reviewed in 25,26).

ANP Synthesis and Secretion from the Heart

ANP Expression During Development and Pathology

The pattern of expression of ANP in the heart varies, depending on the developmental stage, as well as heart health status. ANP is normally expressed in both the atria and ventricles in the embryonic and fetal heart, but soon after birth, ventricular ANP levels decrease considerably, while expression of ANP in the atrium continues to rise with age. For example, in the healthy adult heart, ANP expression in the atria is about 1,000-fold greater than in the ventricles (21). ANP release from the atria can be stimulated by atrial stretch in response to increases in blood volume. In this case, ANP serves as part of an endocrine loop that maintains blood volume over a narrow range. ANP secretion can also be stimulated by numerous neurohumoral substances, such as catecholamines, vasoactive peptides, such as endothelin, and cytokines (27). Since the fetal gene program is reactivated in the ventricles in response to certain pathologies, such as pressure and volume overload hypertrophy, myocardial ischemia, and heart failure (28), as with many other fetal gene program members, expression of ANP in the adult ventricle is often elevated under these conditions (29). However, even at its highest point, ANP expression in the diseased ventricle is relatively low compared to the atria. For example, it has been estimated that only about 30−40% of circulating ANP in heart failure patients is derived from ventricular ANP, while the remainder originates from the atria (30).

Transcriptional Regulation of ANP Expression

Chamber- and development-specific expression of ANP are regulated by a variety of transcription factors (25,26). Prominent among them are GATA-4 and GATA-6, as well as Nkx2.5, MEF-2, Tbx5, SRF and friend of GATA, or FOG-2. These factors appear to be important regulators of ANP transcriptional induction in the fetal ventricle, as well as reactivation of ANP transcription in the diseased heart. The inhibition of ANP expression in the healthy adult ventricle is mediated, at least in part via Hey, which represses GATA-4 and GATA-6-mediated transcription, and Jumonji, which inhibits GATA-4 and Nkx2.5. Additionally, neuron-restrictive silencing factor, or NRSF, binds to a neuron-restrictive silencer element, NRSE, in the ANP gene, and represses transcription in the healthy ventricle. However, in the hypertrophic or failing ventricle, ANP transcriptional repression is relieved, at least partly, via upregulation of a novel NRSF-binding protein, zinc-finger-binding protein 90, Zfp90, which inhibits NRSF-mediated ANP transcriptional repression (31).

Mechanism of ANP Synthesis in the Heart

One of the most intriguing and unusual aspects of ANP is the mechanism of its biosynthesis (Figure 10.1, C). The ANP gene encodes a protein with an N-terminal signal sequence, which is removed co-translationally to give rise to a 126 amino acid form of ANP, called pro-ANP, and, in some species, an initial product of 128 amino acids in length. In those species that express it, the 128 amino acid form of pro-ANP has two C-terminal arginine residues that are removed within the secretory pathway, most likely by a carboxypeptidase known to reside in atrial granules. To this point, the biosynthesis of ANP is typical for a peptide secreted via the classical secretory pathway. However, unlike other peptide hormones, the 126 amino acid form of ANP, often called pro-ANP, is the form of the peptide that is stored in cardiac myocytes, while the circulating form of ANP is a cleavage product of pro-ANP, and is comprised of the C-terminal 28 amino acids of pro-ANP (32).

It remained unclear for some time how and where the conversion of the stored, inactive pro-ANP, to the circulating, active form of ANP took place. It was once hypothesized that the conversion of the storage form of ANP to the circulating form might be the result of processing after its secretion, perhaps in the circulation (33,34). However, pulse-chase labeling experiments using cultured atrial myocytes in serum-free medium (35), as well as experiments with isolated perfused rat hearts that were devoid of blood-borne components, such as proteases (36), demonstrated that the conversion of the 126 amino acid storage form of ANP to the 28 amino acid circulating form took place at the moment of secretion (37) (Figure 10.1, H).

Seven years later, serendipity led to the finding that the type II transmembrane protein, trypsin-like serine protease, now called corin, which, in humans comprises 1,042 amino acids and 116 kDa and is configured with the active site of the enzyme directed toward the extracellular space (38,39), co-secretionally converts pro-ANP to ANP (40,41) (Figure 10.1, H). Thus, it is believed that corin is responsible for the co-secretional maturation of ANP secreted from atrial myocytes in the healthy heart, as well as from ventricular myocytes in the pathologic heart. Support for this belief came from studies on mice in which corin was deleted by gene-targeting; it was shown that ANP was undetectable in the atria of mice that lack corin, and that they develop spontaneous

hypertension, which is exacerbated by a high-salt diet (42). Interestingly, mice lacking corin also exhibited cardiac hypertrophy (42), which is consistent with a role for ANP as an antihypertrophic hormone, which is independent of its effects on blood pressure (43).

Novel Features of Cardiokine Synthesis in the Heart

In terms of secretory protein synthesis and secretion, compared to most other endocrine cells, cardiac myocytes exhibit a number of unique features, suggesting that the heart is an atypical endocrine gland. In addition to distinguishing the heart as an atypical secretory tissue, these features have provided examples of novel roles for the molecular components of the cardiac secretory machinery that could be of importance in other tissues, as well.

Novel Functions for PAM in the Heart

Peptidyl α-amidating monooxygenase, or PAM, has been found in nearly all peptide-secreting tissues examined to date. There are at least two forms of PAM, one form exhibits features of a transmembrane protein, and the other resides in the granule lumen. The tissue exhibiting the highest level of PAM expression is the atrium (44), which is intriguing, because there are no known amidated peptides made in the atrial myocyte secretory pathway, and the only function of PAM known at the time it was discovered in atrial myocytes was peptidyl α-amidation. Thus, it was hypothesized that there might be some yet-to-be-discovered amidated secretory proteins expressed in atrial myocytes. However, to this date, no such proteins have been identified. Moreover, a proteomic analysis revealed the presence of 100 different proteins in highly purified atrial granules, none of which were amidated (45), suggesting that PAM might serve novel functions in the heart. In support of novel functions for PAM in the heart are recent reports demonstrating that under certain conditions, the cytosolic domain of the trans-granule form of PAM is clipped and translocates to the nucleus, where it acts as a transcription factor to regulate genes that are required for secretory granule production (46,47). In other studies it has been shown that pro-ANP, while not a trans-granule membrane protein, is tightly associated with atrial secretory granule membranes, where it binds to the intra-lumenal portion of the trans-granule membrane form of PAM (48), suggesting that PAM and pro-ANP might collaborate to exert an as yet undiscovered function. Further studies have shown that calcium levels in atrial granules are relatively high, that PAM and pro-ANP associate at these levels of calcium in a calcium-dependent manner; moreover, disruption of this association, which requires mutating only two amino acids in pro-ANP, disrupts atrial secretory granule biogenesis, as well as regulated secretion of ANP (49–51). These results suggest that in addition to the role in peptidyl α-amidation, for which it was originally characterized, PAM may collaborate with pro-ANP to facilitate the biogenesis of atrial myocyte granules. In support of this possibility is the finding that in ANP gene-deficient mice, atrial myocytes do not contain secretory granules (52).

Novel Functions for pro-ANP in the Heart

The initial studies showing that pro-ANP is stored in atrial myocytes were carried out before the discovery of the prohormone convertase family of proteases, although it was known that numerous cell types had proteases necessary to process prohormones before secretion. Accordingly, to account for the lack of pro-ANP processing before secretion, it was hypothesized that either atrial myocytes do not express prohormone processing proteases intracellularly, or that if they do, pro-ANP must not be a substrate for those proteases (33,53). Several studies addressed these hypotheses. In the first, pro-ANP was expressed in an endocrine cell line known to process pro-ACTH/β-endorphin, and known not to express pro-ANP (54). In that study, it was shown that pro-ANP was processed prior to secretion, supporting the hypothesis that pro-ANP was not processed prior to secretion in the heart because atrial myocytes do not express prohormone proteases. Following the discovery that the PC family of proteases is responsible for prohormone processin, a proteomic analysis of purified atrial myocytes showed that PCs are not expressed in the atria (45). It was shown that by overexpressing PC1 in cultured atrial myocytes, pro-ANP was efficiently processed prior to secretion (55). Therefore, it became clear that atrial myocytes are unusual among endocrine cells, in that they do not express PCs. While it is not known precisely what roles the storage of pro-ANP serves, it is possible that in atrial myocytes, pro-ANP must be intact in order to function in conjunction with PAM in secretory granule biogenesis.

Novel Mechanism of pro-ANP Processing in the Heart

Pro-ANP is unusual among secreted peptides in that it is co-secretionally cleaved by corin to form the final, circulating products, ANP(1–98) and ANP(99–126) (Figure 10.1, H), the latter of which is often called ANP. Although the precise reasons for this unusual co-secretional processing are not known, it is possible that, in addition to a requirement for pro-ANP in atrial granule biogenesis (see previous sections), there may be other reasons for this unusual processing mechanism. For example, it is possible that regulation of the levels and/or activity

of corin could provide a mechanism by which the rate of release of bioactive ANP from the heart could be fine-tuned. Consistent with this possibility was the finding that treatment of cultured cardiac myocytes with the α_1-adrenergic agonist phenylephrine not only increased pro-ANP, but also increased the amount of corin on the surface of the cultured cells (56). In this same study, corin levels were also elevated in the ventricles of the hearts from mice with heart failure. Another study showed that while the levels of corin were unchanged, or slightly elevated in the hearts from animals or humans with various forms of heart failure, for reasons that are not clear, the activity of corin was reduced in all of the heart failure samples (57,58). These findings correlated with previous findings of increased levels of pro-ANP in the circulation of heart failure patients (59). While many intriguing questions about ANP biosynthesis remain, the discoveries of the co-secretional processing of pro-ANP in 1992 complement the 1999 discovery of corin, a cell-surface protease that can perform this unusual processing event. Future studies will be required in order to fully appreciate the novel aspects of corin-mediated ANP generation in the heart, as well as those aspects that might shed light on mechanisms of the synthesis of other cardiokines that are co-released from the heart with ANP.

NOVEL AUTOCRINE AND PARACRINE SIGNALING PROTEINS IN THE HEART

The Molecular Social Network beyond ANP

To this point, the focus of this chapter has been on using ANP as an example of the expression, synthesis, and secretion of cardiokines. However, since the discovery of ANP, and perhaps fueled in part by that discovery, numerous studies have revealed the existence of many other cardiokines secreted in response to numerous, but nevertheless physiologically relevant, stimuli (60). Most of these cardiokines are expressed and secreted in considerably lower quantities than ANP, which most likely underlies the fact that they have major autocrine and paracrine roles. The discovery of these additional cardiokines has led to the realization that the communication between cardiac myocytes, as well as all of the other cell types in the heart, is crucial for optimal cardiac function. Like ANP, these additional cardiokines are secreted in response to a vast array of environmental cues, including neurohormonal substances, mechanical force or stretch, electrical signals, and changes in oxygen tension.

The remainder of this chapter will focus on the description of several modules of the molecular social network that serves as the basis of communication between cardiac myocytes and other cell types in the heart. In each module, the initiation of communication begins with the sensing of physiologically relevant environmental cues by cardiac myocytes, which respond by secreting cardiokines that transfer information to other cell types in the heart, which themselves secrete additional cardiokines that contribute to the physiological response. Thus, like all social networks, this molecular social network is essential for effective distribution of information, but in this case, it is information integrated by cardiac myocytes in response to environmental cues that is converted to the appropriate physiological response.

Roles of Mechanical Force on Cardiokine Release in the Heart

Hypertension-induced left ventricular hypertrophy alters the genetic program of the myocardium, resulting in structural and functional changes that initially serve as an adaptive ventricular response to pressure overload; but, eventually, these changes result in an increased risk of cardiac failure, sudden death, ventricular dysrhythmias and coronary heart disease (28). While a number of factors, including cardiac ischemia and fibrosis, have been known to contribute to these risks, some of the details of the mechanisms by which cardiokines contribute to the hypertrophic gene program have come to be appreciated more recently. One development that advanced this understanding was the observation that cultured cardiac myocytes undergo hypertrophic growth when they are subjected to mechanical force, or stretch, which mimics the strain they experience, *in vivo,* during hypertension (61). This intriguing finding led to further investigations of how cardiac myocytes convert stretch-induced mechanical force into the biochemical signals that mediate hypertrophic growth. While it had been known that various autocrine and paracrine factors secreted by cardiac myocytes, and other heart-derived cells contribute to hypertrophic growth (62), the link between mechanical force and secretion of the responsible growth factors remained unknown. However, the findings that angiotensin II (Ang II) could be made by cultured cardiac myocytes and fibroblasts, as well as in the heart, *in vivo* (63,64), along with the discovery that it induced cultured cardiac myocyte protein synthesis (65), provided the framework for the seminal studies of Sadoshima et al. (66), who used an *in vitro* model to show that Ang II was secreted by stretched isolated cardiac myocytes, and that it acted in an autocrine manner to induce hypertrophic myocyte growth (Figure 10.2i, A and B). This result not only provided an intriguing function for cardiac myocyte-derived Ang II in cultured cells, and *in vivo,* but, along with the earlier discovery of the intracardiac generation of Ang II (67), it also raised questions about how Ang II could be locally generated in the myocardium.

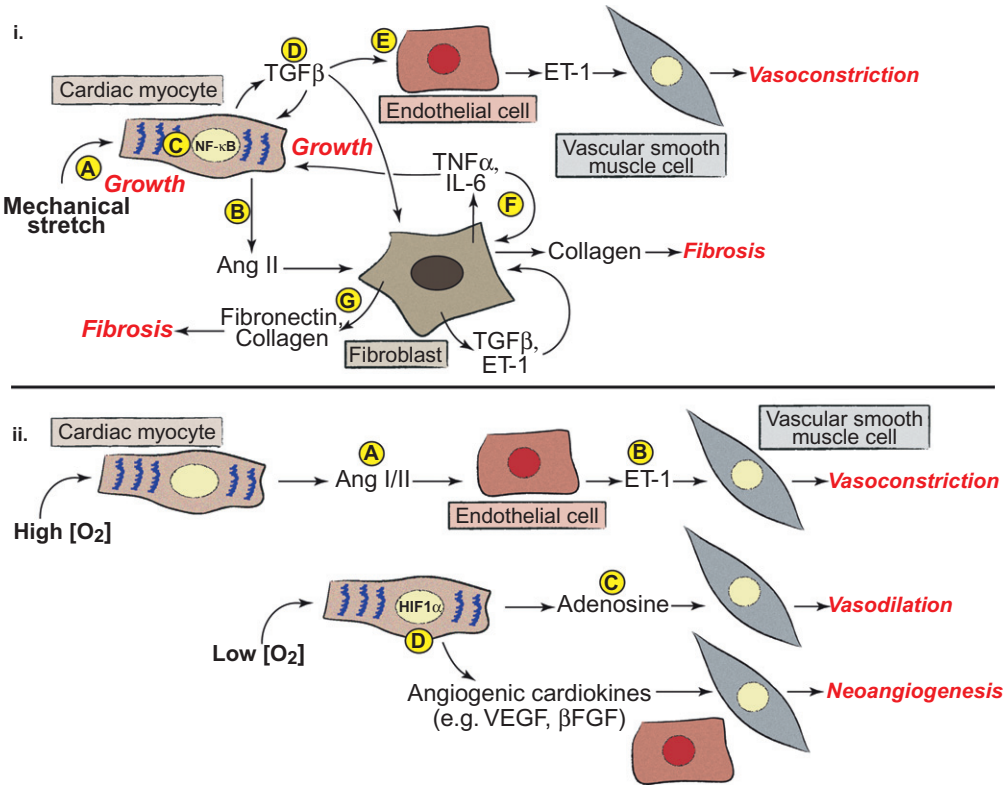

FIGURE 10.2 Panel i: Effects of mechanical force on cardiac cell communication. Mechanical stretch causes the release of Ang II, which has hypertrophic effects on cardiac myocytes (B), and paracrine effects on cardiac fibroblasts (G). Mechanical stretch activates the transcription factor NF-κB, which increases the expression of TGF-β (D). Secreted TGF-β causes the secretion of endothelin (ET-1) from endothelial cells (E). ET-1 causes the contraction of vascular smooth muscle cells. In response to TGF-β, fibroblasts secrete cytokines such as TNF-α and IL-6 (F), which in an autocrine manner activate the fibroblasts, resulting in collagen secretion and fibrosis. TNF-α can act in a paracrine fashion to cause cardiac myocyte growth. **Panel ii: Effects of oxygen tension on cardiac cell communication**. At high oxygen concentrations (one study defines as >6%), cardiac myocytes secrete angiotensin (which can be secreted as Ang II, or converted to Ang II outside of cardiac myocytes) (A). Ang II then causes the secretion of ET-1 from endothelial cells (B), which cause vascular smooth muscle cells to contract, resulting in vasoconstriction. At low oxygen concentrations (defined as <6%), cardiac myocytes secrete adenosine, which directly acts on vascular smooth muscle cells, resulting in vasodilation (C). Activation of HIF1α at low oxygen concentrations results in the upregulation of transcripts encoding, among other proteins, cardiokines that promote angiogenesis.

The classical or systemic renin-angiotensin system (RAS) is well known for its ability to regulate blood pressure and water balance. However, given the required multiple tissues, including liver, kidney, and vascular endothelium for the angiotensin-converting enzyme (ACE)-mediated generation of Ang II in the circulation from a large liver-derived macromolecular precursor, angiotensinogen, the molecular mechanism by which Ang II could be produced locally in the myocardium remained an intriguing mystery for many years. Since that time, additional studies have shown that cardiac myocytes, as well as cardiac fibroblasts, can convert angiotensinogen to Ang II in an ACE-independent, chymase-dependent manner, and that Ang II can be released from these cells via the classical secretory pathway (68). An additional part of the mystery of local Ang II formation in the heart was solved when it was shown that chronic stretch of cardiac myocytes induced angiotensinogen mRNA formation (66). This finding supported the existence of a positive local feedback mechanism between mechanical stretch and cardiac myocyte-derived Ang II.

The intricate roles of Ang II as a key communicator in orchestrating responses of the myocardium to stretch were further appreciated when it was shown that in addition to cardiac myocytes, Ang II exerted diverse autocrine and paracrine effects on other cell types in the heart. For example, Ang II was shown to increase release of transforming growth factor-beta (TGF-β) via the classical secretory pathway from cardiac myocytes (Figure 10.2i, D). Cardiac myocyte-derived TGF-β, in turn, was shown to act in a paracrine manner to increase the expression and constitutive release of the pressor peptide, endothelin (ET-1) from vascular endothelial cells via the classical secretory pathway (69) (Figure 10.2i, E). TGF-β was also shown to

exert paracrine effects on cardiac fibroblasts, where it increased expression and secretion of numerous cardiokines, including IL-6 and TNF-α, both of which were released via the classical secretory pathway (Figure 10.2i, F). In addition, these cardiokines were also shown to contribute to increased collagen expression in cardiac fibroblasts, which is released via the classical secretory pathway, after which its deposition in the extracellular matrix contributes to myocardial fibrosis. In addition to this indirect effect, Ang II can affect fibrosis directly by inducing the expression of numerous extracellular matrix proteins, including fibronectin and collagen in cardiac fibroblasts (70) (Figure 10.2i, G). Moreover, the Ang II-dependent increase in collagen secretion is accompanied by decreased secretion of collagenase, which exacerbates the untoward effects of collagen-mediated fibrosis on myocardial contractility (71). Ang II can also stimulate the secretion of TGF-β and ET-1 from cardiac fibroblasts (72).

Roles of Oxygen Tension on Cardiokine Release in the Heart

Hypoxia is a recurrent theme underlying the pathophysiology of several cardiac diseases, including hypertrophy and ischemic heart disease. In pathological cardiac hypertrophy, the rate of neoangiogenesis does not keep pace with the rate of myocardial growth, which can generate a chronic ischemic state. In the case of myocardial infarction, myocardial ischemia is the result of insufficient coronary blood flow, usually due to atherosclerosis. In both cases, hypoxic stress activates a variety of signaling pathways that are designed, in part, to facilitate adaptation of the heart to changes in oxygen tension (73). Although physiological mechanisms coordinate cardiac oxygen balance dynamically in response to acute alterations in cardiac workload, changes in the expression of genes encoding proteins that regulate coronary blood delivery also play a critical role, generally as adaptive responses to cardiac stressors that alter either myocardial oxygen consumption (e.g. hypertrophy) or oxygen delivery (e.g. coronary artery disease). The sensitivity of the myocardium to oxygen tension has led to studies examining not only how cardiokine secretion is altered under hypoxic conditions, but also how the heart and coronary vasculature respond to changes over the hypoxic to the hyperoxic range of oxygen concentrations. Communication between cardiac myocytes and other cells in the heart has been shown to be the basis of local myocardial adaptation to hypoxia, as well as hyperoxia. For example, Winegrad et al. have shown that when the concentration of oxygen is >6%, cardiac myocytes secrete angiotensin (74) (Figure 10.2ii, A). According to these same investigators, Ang II then causes the secretion of ET-1 from endothelial cells, which interacts with neighboring vascular smooth muscle cells to foster vasoconstriction (Figure 10.2ii, B). However, at oxygen levels <6%, in the same study it was shown that cardiac myocytes release adenosine, which causes vascular smooth muscle cell relaxation, leading to vasodilation (Figure 10.2ii, C). Moreover, adenosine has also been shown to promote angiogenesis by upregulating vascular endothelial growth factor (VEGF)mRNA (75). These are among the mechanisms by which cardiac myocytes can communicate with nearby cells in a paracrine manner to autoregulate vascular tone in response to hypoxia and hyperoxia, thus providing a mechanism for maintaining optimal oxygen levels in the myocardium over a narrow range.

Another mechanism for regulating proper myocardial oxygen perfusion during more chronic hypoxia is the release of cardiokines from cardiac myocytes that promote neoangiogenesis. For example, activation of the transcription factor, hypoxia inducible factor 1α (HIF1α) in cardiac myocytes in response to hypoxia induces genes encoding angiogenic factors, such as VEGF, secreted via the classical pathway, and basic fibroblast growth factor (bFGF), secreted via the non-classical pathway, which collaborate to induce growth of new blood vessels (60,73) (Figure 10.2ii, D). HIF1α also induces other genes that encode proteins involved in blood delivery (e.g. vascular remodeling and erythropoiesis) and metabolism, apoptosis, control of reactive oxygen species, vasomotor reactivity and vascular tone, and inflammation.

Roles of Cardiokines in Heart Failure

The progression of heart failure is dependent on the overexpression of neurohumoral substances, including norepinephrine, angiotensin II, and other cardiokines (76). These factors contribute to disease progression by promoting left ventricle remodeling and eventual cardiac dysfunction. This understanding, coupled with earlier studies of Ang II as a cardiac growth factor, has led to the adoption of therapeutic strategies using ACE inhibitors, angiotensin receptor blockers, and beta-blockers to antagonize the rennin-angiotensin and adrenergic signaling systems in attempts to moderate the untoward effects of heart failure. However, despite these strategies, which may slow the progression of heart failure, there is currently no treatment for resolving heart failure in the long term. Accordingly, there have been numerous studies aimed at identifying the cardiokines that contribute to, or protect from the effects of heart failure, which have been called pro- or anti-inflammatory cytokines, respectively.

Several lines of evidence indicate that, at least in part, the source of heart failure-related cardiokines are cardiac myocytes. Indeed, myocardial overexpression of TNF-α (77–79) was shown to be sufficient to cause heart failure.

Moreover, circulating TNF-α, as well as IL-6 family members, including IL-6, LIF, and cardiotrophin, which may originate from the periphery or the myocardium, increase with the severity of heart failure (80–83). Most of the effects of these cytokines are due to their abilities to bind to gp130, a cell-surface receptor that is upregulated to a greater extent in patients with dilated cardiomyopathy than in those with valvular, or ischemic cardiomyopathy, suggesting that, at least in part, their effects depend upon the etiology of the heart failure.

As illustrated in the "mechanical stretch" section, there is crosstalk between the RAS and cytokine expression and secretion, which may amplify and sustain a positive feedback loop, leading to hypertrophy, cardiac dysfunction, and, eventually, heart failure. This suggests that cytokines may be both a cause and a consequence of heart failure. In addition to cytokine production during pressure overload, myocardial infarction increases production of cytokines such as TNF-α, IL-1β, IL-5, and IL-6 (84,85). Treatment with ACE inhibitors following an infarction decreases cytokine production, and treatment of heart failure patients with AT_1R antagonists has shown a decrease in circulating levels of TNF-α, suggesting the possibility that one of the actions of ACE inhibitors may be through inhibition of cytokine production (86,87). Conversely, cytokines can upregulate components of the RAS, fueling the positive feedback loop, and in some cases making the upregulation of the RAS secondary to the development of the cardiomyopathy (88).

It is important to mention that in heart failure the levels of circulating inflammatory cytokines, such as those mentioned above, are much lower than those observed in inflammatory diseases, such as sepsis (89). Thus, it is possible that these cytokines may exert different effects, depending upon their levels. For example, activation of the immune system with TNF-α or IL-6 can promote survival mediated by the transcription factor, the signal transducer, and activator of transcription 3 (STAT3), which following phosphorylation translocates to the nucleus and the mitochondria, where it exerts protective effects. This pathway, recently discovered in infarct and ischemic heart failure, is known as the survivor activating factor enhancement (SAFE) pathway in the heart (89). Interestingly, the protective aspects of this pathway during ischemic heart failure do not translate to nonischemic heart failure. Additionally, a large number of experimental models and clinical studies have investigated the roles of hematopoietic cytokines, including the inflammatory hallmarks of heart failure mentioned here, in cardiac repair and stem cell recruitment and homing. These studies have also shed light on the potential cardioprotective aspects of cytokine therapy, and have shown that the outcomes depend on timing of therapy, extent of stem cell mobilization, the patient population, and mobilization-independent effects of cytokines (90).

CONCLUSIONS

Since 1981, when the heart was first shown to be an endocrine organ, studies of the autocrine, paracrine, and endocrine mechanisms by which the heart communicates have revealed the existence of a complex network of cardiokines and their receptors. These fundamental elements of a molecular social network provide the basis for all communication of the developing, healthy, and pathological heart. This communication serves as a critical framework for all acute, as well as chronic, responses of the heart to environmental cues. Accordingly, future studies aimed at determining the roles of cardiokines in areas such as myocardial regeneration and the mitigation of damage in the diseased heart may contribute to the development of novel therapeutic approaches.

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Chapter 11

Calcium Fluxes and Homeostasis

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INTRODUCTION

Changes in myocyte $[Ca^{2+}]$ regulate myocyte contraction, metabolism, growth, hypertrophy, and death (1). In this chapter we will review the mechanisms for Ca^{2+} entry and exit from the cytoplasm. We will discuss the pathways for Ca^{2+} influx and efflux across the sarcolemma, for Ca^{2+} uptake and release by the sarcoplasmic reticulum (SR) and mitochondria and for regulation of $[Ca^{2+}]$ in myocyte microdomains, such as the nucleus, where $[Ca^{2+}]$ is thought to be involved in local signaling processes. We will also discuss the role of disrupted $[Ca^{2+}]$ homeostasis in abnormal myocyte contractility and pathological hypertrophy.

Heart muscle imparts energy to the blood to propel it through the blood vessels, thereby providing oxygen and nutrients to the tissues, and removing their metabolic waste. The heart is an intermittent pump that fills with blood when the muscle is relaxed (diastole) and ejects blood into the outflow arterial system when contraction is activated (systole). Contraction and relaxation are controlled by regulation of the cytoplasmic free $[Ca^{2+}]$ ($[Ca^{2+}]_i$). During diastole, $[Ca^{2+}]$ is kept at low levels (below 150 nM) which limits Ca^{2+} binding to the myofilament regulatory site that is responsible for activation of contraction. During systole $[Ca^{2+}]_i$ rises and this activates contractile proteins to produce force and shortening (or pressure and ejection).

Ca^{2+} is the master regulator of contraction and relaxation and it is exquisitely controlled (2). Cardiac myocytes have a sophisticated Ca^{2+} control system that keeps $[Ca^{2+}]$ at low levels during diastole (to ensure a compliant heart that easily fills) and ensures, with a high safety margin, that $[Ca^{2+}]$ is rapidly and uniformly elevated within each myocyte (the $[Ca^{2+}]$ transient) in response to the propagating cardiac action potential. This elevation in $[Ca^{2+}]_i$ promotes Ca^{2+} binding to the thin filament Ca^{2+} binding protein troponin C, to induce contraction. When troponin C binds Ca^{2+} at its regulatory site, other sites on the thin actin filament become accessible to the myosin heads on the thick filament and the acto-myosin ATPase is the motor that transduces chemical energy (ATP) into mechanical energy (contraction; see Chapter 13).

Under basal conditions, the increase in $[Ca^{2+}]_i$ during the $[Ca^{2+}]$ transient is not sufficient to maximally activate the contractile apparatus within the myocyte. Thus, the normal heart has a tremendous capacity to increase the amplitude of the Ca^{2+} transient and thereby increase the activation of the contractile apparatus within the myocytes, causing the strength of cardiac contraction to increase (3). By varying the amplitude and duration of the systolic $[Ca^{2+}]$ transient the heart is able to regulate its pumping capacity (strength) to provide the range of blood flow that is needed to support different levels of tissue metabolism. Aerobic exercise is an example of a physiological condition in which the pumping capacity of the heart is increased by many fold. The ability of the normal cardiac myocyte to change its contractile capacity over a broad range is brought about by two key factors. The first is intrinsic to myofilament geometry, where increased diastolic volume (and sarcomere length) enhances the force of contraction (4). This is known as the Frank–Starling law of the heart, and helps the heart to adjust its output to match the amount of blood that returns to and fills the heart. The second mechanism (and our focus here) is that the amplitude and duration of the systolic $[Ca^{2+}]$ transient can increase and drive a stronger contraction. This pathway is referred to as an increase in contractility or inotropic state (and is distinct from the Frank–Starling mechanism).

Ca^{2+} is also involved in the regulation of other vital cardiac myocyte processes. The heart is an aerobic tissue, and oxygen utilization must be matched by oxygen delivery. At the level of the individual myocyte, ATP utilization must be matched to ATP generation. Ca^{2+} plays a central role in this process, by Ca^{2+}-dependent regulation of mitochondrial ATP synthesis. Myocytes match enhanced cardiac function and metabolism through $[Ca^{2+}]$-dependent regulation of contraction and energy production (5).

Muscle. DOI: http://dx.doi.org/10.1016/B978-0-12-381510-1.00011-9

TABLE 11.1 Ion Concentration and Electrochemical Potentials

	$[X]_o$ (mM)	$[X]_I$ (mM)	E_R (mV)
Na^+	140	10	+70
K^+	5	120	−84
Ca^{2+}	2	0,0001	+131
Cl^-	130	15	−57

**Nernst potential, E_m where the concentration gradient exactly balances the electrical gradient (i.e. no net flux).*

Physiologically, the heart changes size during normal growth, with aerobic exercise training, and with pregnancy. Changes in myocyte $[Ca^{2+}]$ appear to be centrally involved in physiological growth (6). Cardiac diseases, including hypertension or post-myocardial infarction remodeling, result in pathological cardiac hypertrophy, myocyte apoptosis, and myocyte necrosis (7–12) and altered myocyte Ca^{2+} handling can play a role in this maladaptive process.

INTRACELLULAR AND EXTRACELLULAR Ca^{2+} CONCENTRATIONS DURING DIASTOLE

Cardiac myocytes maintain steep transmembrane ion gradients (see Table 11.1). In particular there is a very large $[Ca^{2+}]$ gradient across the surface membrane, with intracellular $[Ca^{2+}]$ (100 nM) being about 20,000 times lower than extracellular $[Ca^{2+}]$ (2 mM). This gradient is established and maintained by active transport mechanisms including Na/Ca exchange and sarcolemmal Ca^{2+} ATPase. The resting myocyte membrane potential (E_m) is negative, so there is also an electrical gradient favoring Ca^{2+} entry. This large inwardly directed electro-chemical energy gradient promotes Ca^{2+} entry through a variety of pathways.

Surface Membrane Ca^{2+} Influx

The major influx pathway for Ca^{2+} entry is through voltage-regulated L-type Ca^{2+} channels (LTCC). These channels are closed at the normal resting (diastolic) E_m and they open, to allow Ca^{2+} to enter, during the cardiac action potential (11). In diastole, in spite of the steep energy gradient for Ca^{2+} entry, Ca^{2+} influx is small because LTCCs are closed, and this allows Ca^{2+} efflux pathways to maintain a low $[Ca^{2+}]_i$. This low diastolic $[Ca^{2+}]_i$ keeps the heart relaxed and thus allows ventricular filling at low pressures. During systole, contraction is activated by a rapid rise in $[Ca^{2+}]_i$ and the Ca^{2+} regulatory processes involved are well understood.

In all cardiac myocytes, the systolic $[Ca^{2+}]$ transient is caused by the influx of a small amount of Ca^{2+} through LTCCs that are activated by depolarization during the upstroke of the action potential (1,13–15). This Ca^{2+} influx induces the release of a much larger amount of Ca^{2+} that has been stored in the sarcoplasmic reticulum (SR). This process is called Ca^{2+}-induced SR Ca^{2+} release (CICR) and is the major (and possibly exclusive) mechanism governing SR Ca^{2+} release in the mammalian heart. LTCCs are the major route of Ca^{2+} entry in the adult heart and the normal activator of SR Ca^{2+} release (16). LTCCs are primarily, but not exclusively, localized to T-tubular invaginations of the surface membrane in close apposition to the junctional regions of the SR. This anatomical arrangement brings LTCCs into close proximity with the SR.

LTCCs and Excitation–Contraction Coupling

Ca^{2+} influx through the LTCC is essential for induction of cardiac contraction. This process takes place in a specialized region of the cardiac myocyte that has been called the couplon (17). There are thousands of couplons within each myocyte and they (Figure 11.1) are mostly along the T-tubules (invaginations of the surface membrane), but a minority are also found at the surface membrane. The typical couplon is made up of a small region of T-tubule membrane (~100–200 nm in diameter), which houses a cluster of LTCCs, a narrow subsarcolemmal cytoplasmic cleft space, and the apposed junctional face of the SR containing Ca^{2+} release channels (ryanodine receptors, RyR2). There may not be a direct molecular contact between the LTCC and RyR in the heart, but there is bidirectional signaling whereby Ca^{2+} entry triggers RyR opening and the Ca^{2+} released by the RyR contributes to inactivation of LTCC current (I_{Ca}) (18).

During diastole, both the RyR and the LTCC are closed, and the local $[Ca^{2+}]$ in the subsarcolemmal cleft space and in bulk cytoplasm is low. The normal cardiac action potential induces the opening of one or more of the LTCCs within the couplon, allowing Ca^{2+} to enter the cleft space and promote Ca^{2+} binding to and activation of the RyR2 (17). The Ca^{2+} from the SR further increases the $[Ca^{2+}]$ in the cleft and promotes the opening of additional RyR channels, a locally regenerative Ca^{2+} release event, and also inactivates the LTCC to limit further Ca^{2+} entry. There is substantial evidence supporting the idea that CICR is the exclusive mechanism for EC coupling in mammalian cardiac myocytes (19). CICR at each of the roughly 20,000 couplons in an individual myocyte are synchronized during every heart beat by the action potential and I_{Ca} (17). There appears to be a high "safety factor" ensuring release at nearly every couplon, because in the normal cardiac myocyte it appears that every

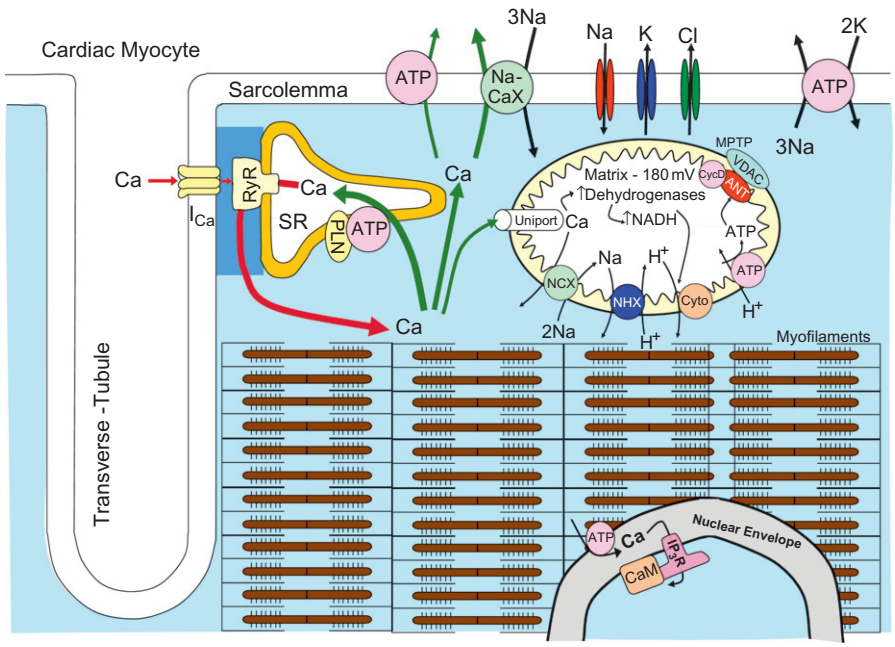

FIGURE 11.1 **Ca^{2+} and other ion transport in cardiac ventricular myocytes.** Ca^{2+} influx occurs via LTCC (I_{Ca}) and removal from the cytosol via the SR Ca^{2+}-ATPase (ATP), Na^+/Ca^{2+} exchange (NaCaX), sarcolemmal Ca^{2+}-ATPase and mitochondrial Ca^{2+} uniport. Mitochondrial Ca^{2+} is extruded via mitochondrial Na^+/Ca^{2+} exchange (NCX), and Na^+ via Na/H exchange (NHX). Cytosolic Ca^{2+} activates the myofilaments. Cyto is cytochromes, IP_3R is IP_3 receptor, Cyclophilin D (CycD), adenine nucleotide transporter (ANT) and voltage-dependent anion channel (VDAC) are potential participants in mitochondrial membrane transition pore (MPTP).

couplon releases its Ca^{2+} with every heart beat. It is important to point out that while SR Ca^{2+} release can be graded experimentally by manipulating the number of LTCCs that open with depolarization (11), this is not the physiological mechanism for changing the amplitude of the normal systolic Ca^{2+} transient. Stated a bit differently, the size of the global [Ca^{2+}] transient can be graded experimentally by changing amplitude of the I_{Ca} and grading the number of couplons that release their Ca^{2+}. However, the high "safety factor" for EC coupling results from the fact that there is a sufficient number of LTCCs at each couplon to ensure that one or more LTCCs opens with each action potential to initiate locally regenerative CICR (17). An important conclusion of much research on this topic is that the physiological grading of the amplitude of the systolic Ca^{2+} transient primarily results from processes that grade the filling of SR Ca^{2+} stores rather than by recruitment of couplons that are normally non-functional.

LTCC Biophysical Properties

The LTCC is a very well-characterized ion channel. Ca^{2+} flux through the LTCC participates in the generation of the cardiac action potential, is essential for normal EC coupling, and activates signaling molecules that regulate metabolism, growth, survival, and death. During diastole, E_m in most cardiac myocytes is near −80 mV, and at this potential the LTCC is closed, but available to open with depolarization (11,15,19). LTCCs begin to activate during the depolarizing upstroke of the cardiac action potential. As E_m increases beyond −40 mV, some LTCCs begin to open and open probability reaches a maximum when E_m reaches about +10 mV. Therefore, the normal cardiac action potential (AP) traverses a voltage range that is sufficient to activate the LTCC (17). However, not all LTCCs open during the action potential. In normal myocytes <15% of the channels open during the action potential (20). This fraction of LTCC is sufficient to cause SR Ca^{2+} release from virtually all couplons (17). LTCC open probability can be increased by protein kinase A (PKA) (21) and Ca^{2+}-calmodulin kinase (CaMKII) (22) phosphorylation cascades (discussed later) and these are important physiological mechanisms to increase Ca^{2+} influx. Since all couplons already are activated to release their stored Ca^{2+}, this additional Ca^{2+} influx regulates cardiac contractility by increasing the SR Ca^{2+} load (23). Diseases that reduce Ca^{2+} influx through the LTCC can negatively impact EC coupling (24), SR Ca^{2+} loading and contraction (25).

LTCCs in Signaling Microdomains

LTCCs are primarily housed in "couplons", the EC coupling microdomain. As discussed above, the function of the LTCCs in these regions of the sarcolemma is to ensure that stored SR Ca^{2+} is released with each heart beat. Some LTCCs are found outside of the couplon, but the function of these channels is not yet fully established. One example is LTCCs that are in caveolae, together with β_2-adrenergic receptors, Ca^{2+}-dependent nitric oxide synthase, the plasma membrane Ca^{2+}-pump, and other signaling molecules (26). The functional role of LTCCs that are housed in discrete portions of the membrane with

other signaling molecules needs clarification. Clearly, signaling that requires brief periods of high local $[Ca^{2+}]$ could require spatially localized LTCC. Given that these channels should open and close like those in EC coupling microdomains, they could be LTCC "activity" signals that trigger downstream changes in metabolism or growth. Microdomain Ca^{2+} signaling is discussed in more detail later in this chapter.

Other Ca^{2+} Conducting Channels

The LTCC is the major Ca^{2+} influx pathway in the heart. However, there are other Ca^{2+} channels that appear to have important functional roles in the normal and diseased heart.

T-type Ca^{2+} Channels (TTCC)

TTCC are primarily expressed in the fetal heart (27,28) where their functional significance is still not well understood. In the developing heart there is robust expression of two of the three TTCC genes (α_1g and α_1h) (28). Expression of TTCC may precede that of the LTCC (28), and this may be an important Ca^{2+} influx pathway to regulate contraction and growth of newly formed myocytes. However, the functional role of these channels is still not well understood. The expression of TTCC decreases after birth and in the normal adult ventricle there is little if any TTCC expression. TTCC are re-expressed in the ventricle with pathological stress (29). Again, the functional role of the Ca^{2+} which enters through these channels is not clearly defined. There are studies suggesting that Ca^{2+} influx through re-expressed TTCC is involved in the induction of cardiac hypertrophy (30), prevention of cardiac hypertrophy (31), and in the generation of new myocytes from cardiac progenitor cells (32). All of these findings need significant additional study. With regard to new myocyte formation, there is evidence in other cell types that expression of TTCC is linked to the cell cycle and cell proliferation (33).

There is strong support for the idea that Ca^{2+} influx through TTCCs is not involved in EC coupling (16). This may be due to the fact that these channels appear to be localized to the surface membrane away from EC coupling microdomains (16). The signaling domains that house TTCC remain to be defined but there is some evidence that Ca^{2+} influx through these channels can activate NO signaling cascades (31).

SURFACE MEMBRANE Ca^{2+} EFFLUX MECHANISMS

To maintain Ca^{2+} homeostasis, the amount of Ca^{2+} entering the myocyte must be balanced by Ca^{2+} efflux. Na^+/Ca^{2+} exchange (NCX) is the Ca^{2+} transporter in heart that is largely responsible for extruding the Ca^{2+} which enters via the LTCC, the major influx pathway. This is an active, energy-utilizing process, with the energy for Ca^{2+} efflux (against its electro-chemical gradient) being derived from the inward movement of Na (down its electrochemical gradient). To maintain the normal energy for NCX requires that the Na/K ATPase (Na pump) maintain a normal intracellular (Na) (1,34). NCX stoichiometry is generally accepted to be $3Na^+:1Ca^{2+}$ (34), and is electrogenic with extrusion of 1 Ca^{2+} coupled to inward flux of 3 Na^+, with one net positive charge. This transport mechanism generates an ionic current (I_{NCX}). I_{NCX} is reversible and its direction and amplitude are controlled by $[Na^+]$ and $[Ca^{2+}]$ on both sides of the membrane as well as E_m. I_{NCX} reverses at $E_{NCX} = 3E_{Na} - 2E_{Ca}$, and this is analogous to a reversal potential for an ion channel current. At rest E_m is negative to E_{NCX} (typically −50 mV), such that Ca^{2+} extrusion is favored thermodynamically, even though the low $[Ca^{2+}]_i$ limits the absolute rate of Ca^{2+} extrusion and diastolic inward I_{NCX}. During the AP upstroke, E_m passes E_{NCX} so that Ca^{2+} influx and outward I_{NCX} is favored (35). However, this period is very brief, because as soon as I_{Ca} is activated and SR Ca^{2+} release ensues, the very high local $[Ca^{2+}]_i$ near the membrane drives E_{NCX} back above E_m such that I_{NCX} becomes inward and extrudes Ca^{2+} again. Notably, the higher the Ca^{2+} transient and the further repolarization proceeds, the greater the inward current. These processes are modified in cardiac disease states and contribute to abnormal $[Ca^{2+}]$ handling (see below). In HF, where SR Ca^{2+} release can be smaller than in normal myocytes, $[Na^+]_i$ is elevated and the APD is prolonged, the situation can be reversed, such that outward I_{NCX} and Ca^{2+} influx occur throughout most of the AP (36).

The NCX is designed to rapidly respond to changes in Ca^{2+} influx to establish a new steady state. For example, if Ca^{2+} influx increases (higher heart rate or sympathetic stimulation) this will increase SR Ca^{2+} loading and release, resulting in an increase in the amplitude of the Ca^{2+} transient. This elevation in time-averaged cytosolic $[Ca^{2+}]$ will increase Ca^{2+} efflux via NCX, so that Ca^{2+} efflux balances Ca^{2+} influx. This is a critical feature of normal Ca^{2+} regulation, because Ca^{2+} influx and SR Ca^{2+} release can change over a broad range, and the NCX is designed to rapidly adapt Ca^{2+} efflux to balance the system (37,38). This allows Ca^{2+} influx and SR Ca^{2+} loading and release to be changed to support a broad range of cardiac contractile function while maintaining Ca^{2+} flux balance. No phosphorylation-dependent NCX regulation is required for this behavior of the NCX.

Ca^{2+} efflux can also take place via an ATP-driven membrane pump (sarcolemmal Ca^{2+} ATPase) (39). This transport pathway is responsible for eliminating a very

small quantity of Ca^{2+} from cardiac myocytes (40). However, given the fact that Ca^{2+} can accumulate in or be depleted from submembrane signaling microdomains, sarcolemmal Ca^{2+} ATPase that is localized in microdomains such as caveolae could play an important role in local $[Ca^{2+}]$ regulation in these specialized regions of the cell. Some experiments in genetically modified mice with gain or loss of sarcolemmal Ca^{2+} ATPase suggest that these transporters control the microdomain $[Ca^{2+}]$ that influences myocyte growth and death signaling (41).

SARCOPLASMIC RETICULUM (SR) Ca^{2+} STORAGE AND RELEASE

Cardiac myocytes are large cells, and synchronous activation of the contractile apparatus requires a system that is able to rapidly deliver and resequester Ca^{2+} to and from the myofibrillar proteins. This allows for the synchronous activation of contraction and relaxation that is needed for optimal contractile efficiency.

In diastole the SR accumulates Ca^{2+} from the cytoplasm via active transport by the SR Ca^{2+} ATPase (SERCA) (42,43). This ion transport protein is found throughout the SR and uses the energy derived from the splitting of ATP to transport Ca^{2+} from the cytoplasm into the SR lumen (Figure 11.1). Large amounts of Ca^{2+} can be stored in this cellular organelle, since it contains large quantities of Ca^{2+} buffering proteins, such as calsequestrin which is largely confined to the terminal cisternae (junctional regions abutting the T-tubules that form the couplon). By the end of diastole in the normal heart, active SR Ca^{2+} transport has recovered the amount of Ca^{2+}released and these stores are available for the subsequent systole.

Ca^{2+} release from the SR is controlled by activation (opening) of SR Ca^{2+} release channels (RyR2), which are the major pathway for Ca^{2+} release flux from the SR. RyRs are normally closed during diastole, and the "leak" rate from the SR is normally low (44–47). The regenerative opening of RyRs within the junctional SR (within the couplon) is the principal process that initiates cardiac contraction.

The cardiac action potential initiates cardiac myocyte contraction by causing the T-tubular LTCCs to open and trigger Ca^{2+} release (as described above). This SR Ca^{2+} release spills out into the bulk cytoplasm, where it binds to thin filament Ca^{2+}-binding proteins to initiate contraction (1,2). Termination of SR Ca^{2+} release is somewhat controversial, and two mechanisms may be involved. The traditional view is that Ca^{2+} released from the SR can induce Ca^{2+}-dependent inactivation by binding to a site on the cytosolic side of the RyR (48,49), functionally analogous to Ca^{2+}-dependent inactivation of LTCCs. More recently, it has become evident that luminal free SR $[Ca^{2+}]$ ($[Ca^{2+}]_{SR}$) also regulates RyR gating such that higher $[Ca^{2+}]_{SR}$ activates RyR (50). This may explain the steep dependence of SR Ca^{2+} leak as a function of $[Ca^{2+}]_{SR}$ (45) and the appearance of arrhythmogenic spontaneous waves of propagating CICR at high SR Ca^{2+} loads. This mechanism may also explain the termination of SR Ca^{2+} release, which shuts off when $[Ca^{2+}]_{SR}$ falls to roughly half of the normal diastolic value level rather than continuing to completion (46,47,51). Thus both the rise in local $[Ca^{2+}]_i$ and fall of $[Ca^{2+}]_{SR}$ may contribute to the closure of RyR and hence termination of release.

SR Ca^{2+} uptake is physiologically regulated by an associated inhibitory protein, phospholamban (PLN). Activation of sympathetic signaling pathways with normal daily activities such as exercise cause protein kinase A (PKA) and Ca^{2+}-calmodulin kinase II (CaMKII)-mediated phosphorylation of PLN at Ser-16 and Thr-17 respectively (52). Both phosphorylation sites cause PLN to more weakly interact with SERCA and this enhances SR Ca^{2+} uptake. PLN phosphorylation state can be graded, allowing fine regulation of SERCA activity. PLN phosphorylation enhances SR Ca^{2+} uptake and since sympathetic stimulation also increases Ca^{2+} influx this results in an increase in SR Ca^{2+} loading and release (52). The net effect is an increase in the amplitude and rate of decline of the systolic $[Ca^{2+}]$ transient, or an inotropic and lusitropic effect.

MITOCHONDRIAL Ca^{2+} REGULATION

Cardiac myocytes are aerobic cells that cannot accumulate an oxygen debt. Therefore, ATP must be generated in proportion to metabolic demand. Mitochondria are the main producers of myocyte ATP and they must have a system to vary their ATP production to match metabolic demand. A system that uses the same changes in cytosolic $[Ca^{2+}]$ that regulate the force of contraction (the major energy-utilizing process in the heart) to control mitochondrial ATP production would help to match energy supply and demand. Micromolar intra-mitochondrial $[Ca^{2+}]$ ($[Ca^{2+}]_{Mito}$) can activate the mitochondrial F_1F_0ATP-synthase (53) and several key mitochondrial dehydrogenases (pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, and NAD-dependent isocitrate dehydrogenase) (54). Thus, mitochondrial Ca^{2+} can provide matching of energy supply and demand. Indeed, when cardiac muscle work is increased, (NADH) abruptly decreases (reflecting ATP consumption), but if the increased workload is associated with higher amplitude or frequency of Ca^{2+} transients, (NADH) recovers, matching the time course of slow rise in $[Ca^{2+}]_{Mito}$. In contrast, if the imposed workload was not Ca^{2+}-dependent, NADH declines, but fails to recover (55,56). These findings suggest that $[Ca^{2+}]_{Mito}$-dependent activation of dehydrogenases may

be important in balancing energy supply to demand when increases in workload mediated by Ca^{2+} occur.

Exactly how myocyte $[Ca^{2+}]_{Mito}$ is regulated is still a subject of much debate. Ca^{2+} can enter mitochondria via a Ca^{2+} uniporter, but the rate is slow under physiological conditions, such that during a normal Ca^{2+} transient only a small amount of Ca^{2+} should enter the mitochondria (40). Therefore, changes in mitochondrial $[Ca^{2+}]$ that affect ATP supply likely involve changes in $[Ca^{2+}]_{Mito}$ that take place over several seconds after a change in beating rate or inotropic state (both of which increase cytosolic $[Ca^{2+}]$). The Ca^{2+} that enters mitochondria with increases in time-averaged cytosolic $[Ca^{2+}]$ must be removed from the mitochondria when the $[Ca^{2+}]_i$ falls, and this occurs via a mitochondrial Na^+/Ca^{2+} antiporter (57–59).

It is important to keep in mind that while mitochondria make most of the myocyte ATP, glycolytic enzymes cluster around both the SR Ca^{2+} ATPase and Na/K ATPase (and the ATP-sensitive K channel (60–62)). This is notable because glycolysis can keep local [ATP] high and local [ADP] low, both of which help these pumps develop high transmembrane $[Ca^{2+}]$ and $[Na^+]/[K^+]$ gradients (as these gradients depend on ΔG_{ATP} and hence on [ADP] $[P_i]$/[ATP]). In pathological states in which blood flow to the heart is reduced, glycolysis can help maintain myocyte ionic homeostasis for a period of time even though mitochondrial ATP synthesis is reduced.

Ca^{2+}, MITOCHONDRIA, CELL DEATH, AND CONTRACTILE DYSFUNCTION

Ca^{2+} fluxes into and out of mitochondria are centrally involved in matching ATP synthesis to metabolic demand. However, in pathological states in which Ca^{2+} homeostasis is disturbed and there is often increased cytoplasmic $[Ca^{2+}]$, mitochondrial $[Ca^{2+}]$ overload can cause or contribute to the activation of cell death signaling and/or contractile dysfunction.

Myocyte and particularly mitochondrial Ca^{2+} overload is involved in both necrosis and apoptosis. When myocytes are overloaded with Ca^{2+} (e.g. in reperfusion after ischemia), it can promote activation of hypercontracture, Ca^{2+}-dependent proteases (calpains), and arrhythmias. Mitochondria (which occupy ~35% of cell volume) can temporarily take up large amounts of Ca^{2+}, thereby limiting these undesirable effects. Importantly, excess mitochondrial Ca^{2+} uptake can cause reduced ATP production, if mitochondrial membrane potential is used to drive uncoupled Ca^{2+} influx rather than for proton influx that is coupled to ATP synthesis. In the extreme case, Ca^{2+} uptake can depolarize mitochondria entirely. Therefore, while mitochondria can serve as temporary short-term Ca^{2+} sinks in times of Ca^{2+} overload, this can deprive the cell of necessary ATP and doom the cell to death from either apoptosis (63) or necrosis (9).

High $[Ca^{2+}]_{Mito}$ activates the mitochondrial permeability transition pore (MPTP) and allows molecules up to ~1500 Da to flow freely across the mitochondrial membranes. This will immediately dissipate the mitochondrial membrane potential and release Ca^{2+} to the cytosol, since the mitochondrial electrical gradient can drive $[Ca^{2+}]_{Mito}$ to be higher than $[Ca^{2+}]_i$. This exacerbation of cytosolic Ca^{2+} overload will produce hypercontracture, ATP depletion and cell death. For Ca^{2+}-overload-induced cell death, what may distinguish necrosis from apoptosis is simply whether the cell runs out of ATP first (necrosis) vs. sustaining sufficient ATP to follow the apoptotic pathway. The MPTP opening also uncouples oxidative phosphorylation and causes swelling of the mitochondrial matrix volume and consequent rupture of the outer mitochondrial membrane. This allows mitochondrial cytochrome C release and contributes to caspase activation and apoptosis (9).

Ca^{2+}-dependent proteases (calpains) may also be activated during conditions in which diastolic $[Ca^{2+}]$ is persistently elevated. Calpains can cleave key proteins such as the key myofilament protein troponin I and others, and this may be involved in contractile dysfunction associated with myocardial stunning (64,65). In ischemia–reperfusion calpain also cleaves the pro-apoptotic BH3-only Bcl-2 family member, Bid, which can trigger mitochondrial cytochrome c release, and apoptosis (66).

Ca^{2+} AND TRANSCRIPTIONAL REGULATION AND HYPERTROPHY

Ca^{2+}-dependent signaling is involved in transcriptional regulation of genes that are central to pathological hypertrophy via two major pathways: Ca^{2+}-CaM-CaMKII-HDAC (histone deacetylase) and Ca^{2+}-CaM-Calcineurin-NFAT (nuclear factor of activated T cells (67–70); Figure 11.2). Both of these pathways are activated with persistent pathological cardiovascular stress, such as hypertension and after myocardial infarction. The source of Ca^{2+} to activate these pathological signaling pathways is still not yet fully defined (7,29,71,72).

Ca^{2+}/CAM-CAMKII-HDAC PATHWAY

Elevation of cytosolic $[Ca^{2+}]$ can lead to activation of CaMKII and phosphorylation of its target proteins. Once Ca^{2+}-CaM activates CaMKII, the activated state can be reinforced by autophosphorylation or by oxidization (73) to produce an autonomous CaMKII, active even when $[Ca^{2+}]_i$ declines. These forms of CaMKII are enhanced in

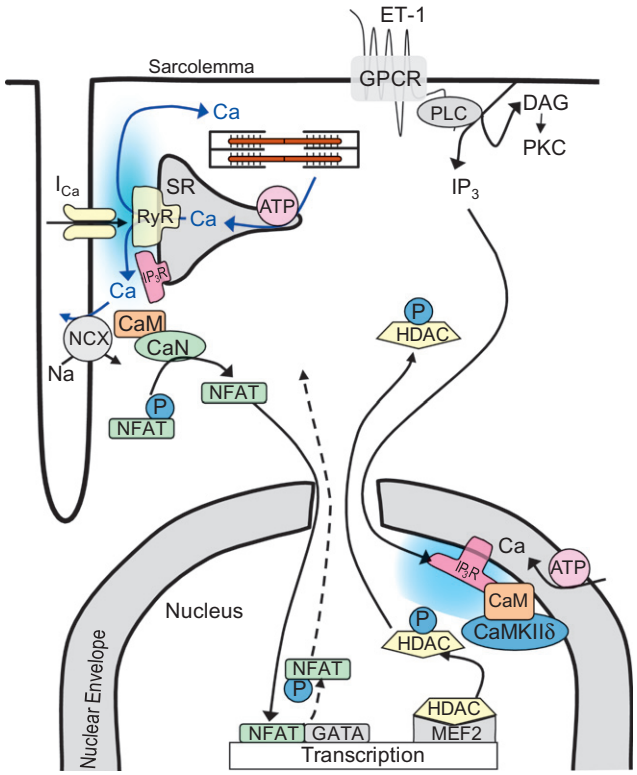

FIGURE 11.2 **Ca^{2+}-dependent processes involved in transcriptional regulation and excitation–contraction coupling (*top left*, as in Figure 11.1).** G-protein-coupled receptors (GPCR) including endothelin-1 (ET-1) or α-adrenergic receptors can all activate phospholipase C (PLC) to produce diacylglycerol (DAG) (which can activate PKC) and IP_3. Local Ca^{2+} release at the nuclear envelope can activate CaMKII, resulting in histone deacetylase (HDAC) phosphorylation (which also occur via PKD). The consequent nuclear export of HDACs relieves HDAC-dependent suppression of MEF2-driven transcription. Calcineurin (CaN) is activated by Ca-CaM and can dephosphorylate NFAT which is then translocated to the nucleus, where it can activate the transcription factor GATA contributing to hypertrophic gene transcription.

several dysfunctional conditions (73). While CaMKIIδ is the predominant isoform in cardiac myocytes, there are splice variants which include a nuclear localization sequence or not ($CaMKII\delta_B$ and $_{C}aMKII\delta_C$) and also minor levels of CaMKIIβ and γ isoforms (73). Therefore, CaMKII regulation of function and transcription is complex.

CaMKII has been implicated in regulation of various transcription factors, e.g. activation protein 1 (AP-1), activating transcription factor (ATF-1), serum response factor (SRF), cAMP-response element binding protein (CREB), and myocyte enhancer factor 2 (MEF2) (74). CREB can be phosphorylated by CaMKII and CaMKIV, but this was unaltered in transgenic mice overexpressing $CaMKII\delta_B$ or CaMKIV (68,75). This implied that CREB was not critical for CaMKII-dependent cardiac hypertrophy. In contrast, CaMKII-dependent MEF2 activation is strongly implicated in hypertrophy (68), but not by phosphorylating MEF2. Rather, the CaMKII–MEF2 link seems to be via phosphorylation of class II HDACs (70,76). Activation of nuclear CaMKII can activate hypertrophic genes by causing the export of those HDACs that normally repress the expression of these genes.

Class II HDACs (HDAC4, 5, 7, and 9) are expressed in heart and have a MEF2-binding domain in an N-terminal extension that is not present in other HDACs (76). This N-terminal region contains two conserved serines that can be phosphorylated by CaMKII (or the related protein kinase D, PKD), facilitating association with the chaperone 14-3-3, and export of the complex from the nucleus (74,76). This relieves MEF2 repression and allows transcriptional activation. HDAC activity is opposed by histone acetyl transferases (HATs), which compete with HDACs at MEF2-binding sites, and acetylate histones, relaxing chromatin structure and facilitating transcription. While Ca^{2+}/CaM can bind to the MEF2-binding domain of HDACs and disrupt the MEF2-HDAC complex, phosphorylation of HDAC is thought to be more important.

Key fundamental mechanisms regarding G-protein-coupled receptor dependent regulation of HDAC4 and HDAC5 phosphorylation have been elucidated by Olson's group in Cos cells and cultured neonatal rat ventricular myocytes (75–77). HDAC4, in particular, directly binds CaMKII, giving CaMKII preferential access to activate HDAC4 nuclear export, making HDAC4 a key CaMKII-dependent regulator of hypertrophy (76). Indeed, CaMKII-dependent HDAC4 phosphorylation causes hypertrophy, while overexpression of HDAC4 limits agonist induced hypertrophy. Details of how specific Ca^{2+} signals are involved in this CaMKII-dependent HDAC4 phosphorylation are lacking. In their cellular expression systems HDAC5 did not seem to be activated by CaMKII, but rather by PKD, in response to both endothelin-1 and phenylephrine. Moreover, for phenylephrine, PKC was responsible for PKD-dependent HDAC5 phosphorylation, while for endothelin-1 PKD activation appeared to be PKC-independent (77).

The Bers lab has studied HDAC5 translocation in adult rabbit ventricular myocytes and found that endothelin-1-induced HDAC5 nuclear export depended equally on CaMKII and PKD (72). Part of the difference may be that myocyte PKD expression dramatically decreases as neonatal myocytes mature to adult myocytes (78), and CaMKII might have greater access to HDAC5 in adult myocytes. The Ca^{2+} signaling involved in endothelin-1-induced HDAC5 phosphorylation in adult ventricular myocytes required $InsP_3$, the type 2 $InsP_3$ receptor (which associates with CaMKII at the nuclear envelope) (79), CaM and Ca^{2+} release from stores (72). However, HDAC5 phosphorylation was completely insensitive to either the cytosolic or nuclear Ca^{2+} transients associated

with E-C coupling (72). They concluded that endothelin-1 induced $InsP_3$ production and diffusion to the nuclear $InsP_3$ receptor, causing locally high $[Ca^{2+}]_i$ and activation of local CaMKII, which can then phosphorylate HDAC5. This allows myocytes to distinguish different local vs. global Ca^{2+} signals that mediate different signaling pathways in cardiac myocytes.

Ca^{2+}-CAM-CALCINEURIN-NFAT PATHWAY

Activation of the phosphatase calcineurin (phosphatase 2B) by Ca^{2+}-CaM is thought to be centrally involved in pathological cardiac myocyte hypertrophy. Calcineurin dephosphorylates NFAT, causing NFAT import into the nucleus where it works cooperatively with cardiac-restricted zinc finger transcription factor GATA4 to activate transcription of hypertrophic genes (80). Cardiac specific overexpression of activated calcineurin or NFAT causes hypertrophy and heart failure, while calcineurin inhibition (using several strategies) can inhibit pathological hypertrophy (67,80–82). Knockout of NFATc3 (but not NFATc4) could inhibit hypertrophy in response to aortic banding, angiotensin II infusion or calcineurin overexpression (83).

Calcineurin has much higher Ca^{2+}-CaM affinity than does CaMKII ($K_m \sim$ 0.1 vs. 50 nM). Thus, CaMKII may respond to high amplitude or frequent local Ca^{2+} oscillations and may require autophosphorylation for true signal integration. In contrast, calcineurin may better sense smaller sustained $[Ca^{2+}]_i$ elevations. The low off-rate allows intrinsic signal integration (84,85).

There is relatively little data in adult cardiac myocytes regarding the specific Ca^{2+} signals that may activate this system. In some lymphocyte studies sustained global $[Ca^{2+}]_i$ elevation seems key in activating NFAT signals (84) while in others $[Ca^{2+}]_i$ oscillations are more efficient NFAT activators (86,87). In hippocampal neurons L-type Ca^{2+} current induces NFAT translocation (88). In skeletal muscle, electrical stimulation with patterns typical of slow (but not fast) twitch muscle caused a calcineurin-dependent NFATc1 translocation to the nucleus (89). Recently Rinne et al. (90) examined dynamic NFAT translocation in adult cardiac myocytes. NFATc1 was nuclear at baseline in atrial and ventricular myocytes, but lowering $[Ca^{2+}]_i$ could shift it more cytosolic. NFATc3 was more cytoplasmic at baseline, but could be driven nuclear by angiotensin II, endothelin-1, and in heart failure myocytes. Further work will be required to dissect the specific local spatiotemporal Ca^{2+} signals involved in cardiac myocyte calcineurin and NFAT translocation.

Another new Ca^{2+}-related transcriptional regulatory pathway has been described, involving a cleaved C-terminal fragment of the LTCC (91,92). Reducing $[Ca^{2+}]_i$ causes reduced nuclear translocation of this fragment, while Ca^{2+} current activity and elevated $[Ca^{2+}]_i$ enhance nuclear localization. In the nucleus this fragment appears to regulate transcription, including of the LTCC itself, such that when I_{Ca} is low, this feedback system may increase transcription of new LTCCs.

These Ca^{2+}-CaM-dependent excitation–transcription coupling pathways may alter transcription of key Ca^{2+} transport and regulatory proteins such as SERCA, PLB, NCX, LTCC, RyR2, CaM, and CaMKII. This might be part of a long-term feedback loop, where altered Ca^{2+} signaling triggers the altered expression of genes which may feedback to normalize cardiac myocyte function, or contribute to exacerbation of hypertrophic or heart failure phenotypes.

A major unanswered question in this field is the source of Ca^{2+} to activate the signaling cascades that produce pathological hypertrophy (93). There is evidence for a role for Ca^{2+} influx through L- and T-type Ca^{2+} channels as well as for TRP channels (71). If and how these Ca^{2+} influx pathways activate hypertrophic signaling pathways independently of the global systolic Ca^{2+} transient is a conundrum that has not been easily solved.

Ca^{2+} FLUX BALANCE IS Ca^{2+} HOMEOSTASIS

The normal cardiac myocyte has the capacity to vary its contractile function, dependent on the moment-to-moment needs of the heart to pump blood. In times of low metabolic demand (sleep), myocyte contractility is low, while during aerobic exercise myocyte contractility is quite high. Changes in myocyte $[Ca^{2+}]$ are largely responsible for normal alterations in cardiac contractility (37,38). In each specific setting, the myocyte must establish a steady state Ca^{2+} flux balance. In general, conditions in which the myocyte exists in a low contractility state involve lower Ca^{2+} influx and efflux than those conditions where myocyte contractility is high (37,38). The key factor in each situation is that there must be Ca^{2+} flux balance. That is, within the cardiac cycle the amount of trans-sarcolemmal Ca^{2+} influx must equal the amount extruded, and the amount released by the SR must be reaccumulated by the SR. Only during transitions from one steady state to another is there a transient period of Ca^{2+} flux imbalance, and this situation is essential for arriving at a new steady state (see below).

PHYSIOLOGICAL REGULATION OF Ca^{2+} TO CONTROL CARDIAC CONTRACTILITY

The normal heart has a tremendous ability to increase its contractility. In large part this results from activation of the sympathetic reflex mechanisms that are involved in

blood pressure regulation and in the response to changes in activity. Here we will briefly review how normal cardiac myocytes increase their contractility.

Sympathetic neurohormones (norepinephrine and epinephrine) bind to β-adrenergic receptors and ultimately increase cellular cAMP and activate protein kinase A (PKA). PKA phosphorylates a number of Ca^{2+} regulatory proteins to produce an increase in the [Ca^{2+}] transient and contraction. Three key Ca^{2+} regulatory proteins that are PKA phosphorylated are the L-type Ca^{2+} channel complex (21), phopholamban (PLN) (52), and the ryanodine receptor (RYR) (94). PKA phosphorylation of the LTCC increases its open probability, to increase the number of LTCCs that open during the action potential and increase Ca^{2+} influx (95). PKA phosphorylation of PLN removes its inhibitory effect on Ca^{2+} uptake by the SR Ca^{2+} ATPase, resulting in increased SR Ca^{2+} uptake and storage (52). These two effects are largely responsible for the increase in the amplitude of the Ca^{2+} transient, and contractility, induced by catecholamines. PKA phosphorylation of the RyR2 is thought to have an effect on its opening probability, but this effect has not been uniformly observed (95,96). Even if PKA phosphorylation of RyR increases the Ca^{2+} sensitivity of the RyR, this effect will have little independent effect on the amplitude of the Ca^{2+} transient because RyRs are part of a regenerative event at the couplon that activates RyRs and because of what has been termed Ca^{2+} transient autoregulation (37,38). That is, an increase in SR Ca^{2+} release will lead to increased $[Ca^{2+}]_i$ which will drive enhanced extrusion via NCX, which will in turn reduce SR Ca^{2+} content and SR Ca^{2+} release back to the same level as before sensitization.

When the sympathetic nervous system is activated there is first an increase in Ca^{2+} influx through the LTCC, and Ca^{2+} influx temporarily exceeds Ca^{2+} efflux. The activated SR takes up additional Ca^{2+} and this results in increases in SR Ca^{2+} stores (95). Over a few beats the amplitude of the Ca^{2+} transient increases and this increases Ca^{2+} efflux, until the myocyte returns to a flux balance state. Increasing Ca^{2+} influx through the LTCC and increasing Ca^{2+} efflux through the NCX, with an increase in the amplitude of the Ca^{2+} transient are essential for maintaining Ca^{2+} homeostasis over a broad range of Ca^{2+} activation states.

DYSREGULATED Ca^{2+} AND CARDIAC DYSFUNCTION IN HEART DISEASE

Heart failure is a syndrome in which the heart is unable to pump an adequate amount of blood to meet the needs of the tissues (97). This is a complex problem and here we will briefly discuss the changes in myocyte Ca^{2+} homeostasis associated with HF. The failing heart usually is pumping blood against a higher than normal stress (increased systolic wall stress (98)). Therefore, the myocytes are developing higher than normal forces to eject smaller amounts of blood. This condition is chronic, and the high stress state requires persistent activation of compensatory neurohormonal systems (the sympathetic nervous system and the renin-angiotensin system). These persistent stressors promote myocyte hypertrophy and alterations in myocyte Ca^{2+} homeostasis.

Myocytes removed from failing hearts usually exhibit depressed Ca^{2+} "handling" (25). The major changes that have been observed include a reduced density of LTCCs, with increased activity of the LTCCs that are present (99), reduced abundance of SERCa2 (100), reduced PLN PKA phosphorylation (101), increased NCX activity (25), and increased PKA and CaMKII-mediated phosphorylation of RyR2 (102–104). These changes tend to reduce the amplitude and increase the duration of the systolic [Ca^{2+}] transient and can also increase diastolic [Ca^{2+}]. It is currently unclear if these changes in Ca^{2+} homeostasis are causes or consequences of HF. If they are causally related to HF then correcting them should provide improved function. If on the other hand they are effects of HF and occur to limit the Ca^{2+} loading required for persistent generation of high wall stress, then increasing [Ca^{2+}] might be harmful. The fact that many recent clinical trials with inotropic drugs have failed (105) while treatments that limit systolic [Ca^{2+}] (β-adrenergic receptor antagonists) (106,107) have some benefit and suggest that increasing myocyte [Ca^{2+}] in the failing heart should be done cautiously (108).

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