ANTIOXIDANTS & REDOX SIGNALING
Volume 9, Number 4, 2007

DOI: 10.1089/ars.2006.1446

Comprehensive Invited Review

Redox Control of Calcium Channels: From Mechanisms to Therapeutic Opportunities

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ABSTRACT

Calcium plays an integral role in cellular function. It is a well-recognized second messenger necessary for signaling cellular responses, but in excessive amounts can be deleterious to function, causing cell death. The main route by which calcium enters the cytoplasm is either from the extracellular compartment or internal

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stores via calcium channels. There is good evidence that calcium channels can respond to pharmacological compounds that reduce or oxidize thiol groups on the channel protein. In addition, reactive oxygen species such as hydrogen peroxide and superoxide that can mediate oxidative pathology also mediate changes in channel function via alterations of thiol groups. This review looks at the structure and function of calcium channels, the evidence that changes in cellular redox state mediate changes in channel function, and the role of redox modification of channels in disease processes. Understanding how redox modification of the channel protein alters channel structure and function is providing leads for the design of therapeutic interventions that target oxidative stress responses. *Antioxid. Redox Signal.* 9, 409–435.

I. INTRODUCTION

Ion channels underlie the electrical activity of cells. Calcium channels have a unique functional role, because not only do they participate in this activity, they form the means by which electrical signals are converted to responses within the cell. Calcium concentrations in the cytoplasm of cells are maintained at a low level, and calcium channels activate quickly such that the opening of ion channels can rapidly change the cytoplasmic environment. Once inside the cell, calcium acts as a 'second messenger' prompting responses by binding to a variety of calcium-sensitive proteins. Calcium channels play an important role in stimulating muscle contraction, in neurotransmitter secretion, gene regulation, activating other ion channels, controlling the shape and duration of action potentials, and many other processes. Since calcium plays such an important functional role, and since excessive quantities can be toxic, its movement is tightly regulated and controlled through a large variety of mechanisms.

Oxygen is the substrate for the generation of reactive species that play an important role in modulating cell function. Reactive oxygen species (ROS) are, generally speaking, oxygen molecules in different states of reduction. Although once thought to play a role primarily in inducing cell death, ROS such as hydrogen peroxide are believed to interact with cell signaling pathways by way of modification of key thiol groups on proteins that possess regulatory functions. One of these proteins is the calcium channel.

In this review we look at what is known about redox modification of calcium channels and the functional implications. We have first outlined the variety of different calcium channels that are utilized in biological organisms, highlighting their differences, nomenclature and function, and recent research. Next we give an overview of the role of calcium in stimulating muscle contraction, neurotransmitter secretion, and controlling electrical excitability. We then discuss the structure of different calcium channels, highlighting possible redox sensitive sites and the experimental evidence for redox modification of calcium channels and transporters. The regulation of calcium channel function by reactive oxygen species and how this occurs during pathological states is examined. Finally, we present opportunities for drug design in therapeutics. Because this is an emerging area and still relatively young in terms of understanding how redox modifications of the channel can alter function, we hope that the overview of structure and function and where the field is now in terms of roles of calcium channels in disease states will stimulate further research and insights into mechanisms for modifying redox-dependent responses.

II. TYPES OF Ca^{2+} CHANNELS

To understand how calcium signaling can be altered by redox control, it is important first to summarize our knowledge of the proteins that control calcium movement. There are a variety of calcium channels that respond to differing stimuli and carry out a number of physiological roles. Broadly speaking, calcium channels are distinguished into two classes: those that are voltage activated, and those that respond to the binding of calcium or other agonists to allow conduction of calcium ions or release calcium from intracellular stores. Here we introduce the different types of Ca^{2+} channels, noting the names applied to them and the functions they fulfil.

A. Voltage-gated channels

Most cell types express voltage-gated Ca^{2+} channels that play an integral role in calcium influx and regulate intracellular processes such as contraction, secretion, neurotransmission, and gene expression. They are members of a gene superfamily of transmembrane ion channel proteins that includes voltage-gated K^+ and Na^+ channels. Ca^{2+} channels share structural similarities with K^+ and Na^+ channels in that they possess a pore-forming α_1 subunit comprising four repeats of a domain with six transmembrane-spanning segments that include the voltage-sensing S4 segment and the pore forming (P) region. The α_1 subunit is large (190–250 kDa) and incorporates the majority of the known sites regulated by second messengers, toxins, and drugs. This subunit is usually complexed with at least three auxiliary subunits, α_2, δ, β, and γ, with the α_2 and δ subunits always linked by a disulfide bond (Fig. 1).

Nomenclature for the Ca^{2+} channels has evolved over the decades. Initially Ca^{2+} channels were classified according to their ability to be activated by a large or small depolarization and whether the response was rapid or slow and persistent. For example, L-type Ca^{2+} channels are a member of the HVA or high voltage activation type because the channels are activated by strong depolarizations, typically to 0 or +10 mV and are long-lasting in that they are slow to inactivate. In addi-

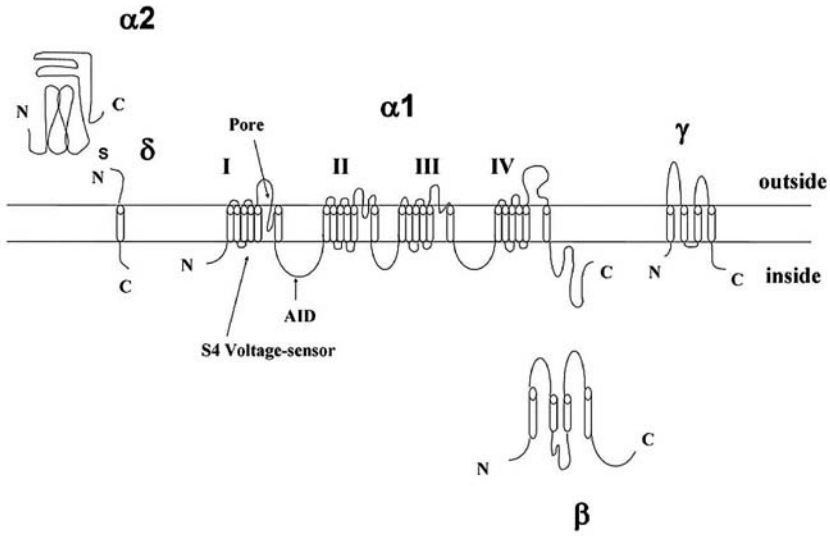

FIG. 1. Subunits of Ca_v channels showing the four homologous domains of the α_1 subunit, and auxiliary β, γ, and $\alpha_2\delta$ subunits. Predicted alpha helices are depicted as cylinders. AID, alpha interacting domain where the β subunit is proposed to interact with the α subunit (see text).

tion, they are blocked by the lipid-soluble 1,4-dihydropyridines such as nifedipine and the (+)-ve enantiomer of Bay K8644 (the (-)-ve enantiomer has agonist properties). T-type Ca^{2+} channels, on the other hand, are transiently activated at low membrane potentials and are insensitive to dihydropyridines and fall into the class of low voltage activated (LVA) channels. Taking into consideration the functional and pharmacological differences, Tsien and colleagues (258) devised a nomenclature based on single letters that continues to be used today. In addition to the L-type (long-lasting currents) and T-type (transient currents) channels mentioned above, a number of other channel types were found with single channel conductances between T- and L-types that were resistant to the dihydropyridines. These channels were predominant in neurons and could be blocked by the ω-conotoxin GVIA from cone snails. They were named N-type (191). P-type (161), Q-type (210), and R-type Ca^{2+} channels have been identified and characterized according to activation and inactivation characteristics and sensitivity to toxins (211).

Channel types were then classified according to distinct hybridization patterns of mRNA on Northern blot analysis and grouped as A, B, C, or D (235). Genes subsequently identified were classified E through I except the skeletal muscle isoform that was referred to as α_{1S} (29). In 2000, Ertel *et al.* (75) suggested a nomenclature based on that used to classify K^+ channels, with Ca as the permeating ion followed by the physiological regulator v for voltage in subscript and the gene subfamily in order of discovery. The Ca_v1 subfamily comprises the L-type Ca^{2+} currents, Ca_v2 subfamily comprises the P/Q, N, and R type Ca^{2+} currents, and Ca_v3 subfamily the T-type Ca^{2+} currents as shown in Fig. 2. Detailed listings of voltage-dependent Ca^{2+} channel nomenclature as approved by the Nomenclature Committee of the International Union of Pharmacology, including structure–function relationships, are available (47, 48).

Although the main characteristics that define channel subtypes such as ion selectivity, voltage dependence, and drug binding sites reside in the alpha subunit, the auxiliary subunits also play important regulatory roles. The first β subunit to be identified (now termed $Ca_v\beta1a$) was cloned from skeletal muscle and observed as a 54 kDa protein (246). To date, four genes have been cloned encoding human β subunits ($Ca_v\beta1$–4) and there are several splice variants produced from the genes (for review, see Refs. 30 and 61). $Ca_v\beta$ subunits are intracellularly located and bind the α subunit with high affinity via the α interaction domain (AID) on the I-II linker (See Fig. 1) (207). $Ca_v\beta$ subunits promote functional expression of $Ca_v\alpha$ subunits, localization, and insertion of the channel complex in the plasma membrane (28, 55). In addition, $Ca_v\beta$ subunits can modify the kinetics and amplitude of current, including increasing the channel open probability and altering the inactivation rate (56).

The $\alpha_2\delta$ subunit is highly glycosylated and encoded by a single gene. The δ segment spans the plasma membrane and connects to the extracellular α_2 protein via a disulfide bridge formed between a number of cysteines present on both pro-

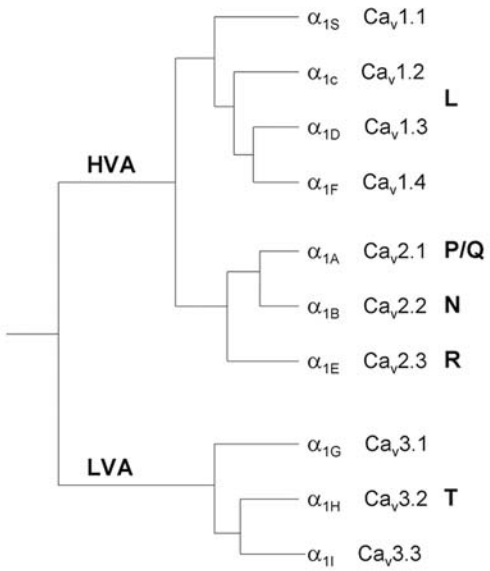

FIG. 2. Family tree of mammalian voltage-gated Ca^{2+} channels and nomenclature. Adapted from Jones (131).

teins. To date, four genes have been identified that encode $\alpha_2\delta 1$–4 subunits (74, 145, 208). The α_2 protein has been shown to influence channel stimulation and the δ protein alters voltage-dependent activation and inactivation kinetics (86).

In some tissues a fourth auxiliary subunit is expressed, the γ subunit. At least eight genes are known to encode for γ subunits ($Ca_v\gamma 1$–8), the first known subunit being cloned from skeletal muscle with a mass of 25 kDa (128). Co-expression studies reveal the subunit can modulate peak current and activation/ inactivation kinetics (230).

B. Ca^{2+} release channels—ryanodine receptor and IP_3 receptor

The Ca-release channels that are responsible for the release of calcium from intracellular stores are inositol triphosphate receptors (IP_3R) and ryanodine receptors (RyR). IP_3Rs and RyRs serve to release calcium into the cytoplasm from the sarcoplasmic reticulum and endoplasmic reticulum of muscle, but they are also expressed in many other cell types including neurons where they play a role in calcium-dependent neurotransmission. In skeletal and cardiac muscle, the activation of IP_3Rs and RyRs is an important step in the process of muscle contraction, allowing intracellular calcium levels to increase and bind to troponin C that then switches on the contractile apparatus. During the course of a single muscle contraction, free intracellular calcium increases from resting concentrations (typically 10–300 n*M*) to micromolar levels (44). In smooth muscle, calcium is bound to calmodulin and phosphorylation of myosin II by Ca^{2+}/calmodulin-dependent myosin light-chain kinase is necessary for intitiation of contraction (183).

Three receptor types or isoforms of RyR and IP_3Rs have been isolated and combinations of isoforms are co-expressed in different muscle types (88, 184). RyRs are localized within membranes of organelles with stores of calcium (such as the sarcoplasmic reticulum), but IP_3Rs have been identified in the plasma membrane, endoplasmic reticulum, Golgi apparatus, and nuclear membranes (139, 153). There is good evidence that both RyR and IP_3Rs share common calcium stores and can interact with each other (176). Activation of both receptor types is Ca^{2+} dependent and varies with isoform. RyRs are activated at micromolar concentrations of calcium while IP_3Rs are generally activated at submicromolar concentrations (27). Therefore, the local release of calcium by one receptor can be amplified by activating a nearby receptor. The dependence of activation on calcium concentration, however, is often bell-shaped so that high concentrations of calcium also have inhibitory effects on channel function. This is the case for type I IP_3Rs where it has been proposed that the bell-shaped-dependence supports oscillations in cytosolic calcium, but not for type III IP_3Rs where an increase in cytosolic calcium causes a further increase in calcium that is suited to signal initiation (108). Both IP_3Rs and RyRs exhibit a high divalent versus monovalent ion selectivity (P_{Ca}/P_K of approximately 7) but have a higher ionic conductance for monovalent cations over divalent cations (26, 160, 234, 254). IP_3Rs mediate calcium release in response to activation of G protein or tyrosine kinase-coupled plasma membrane receptors. Agonist binding leads to stimulation of phospholipase C, resulting in production of diacylglycerol, an activator of protein kinase C, and IP_3. IP_3 is a highly diffusible second messenger that binds to IP_3Rs on membranes of intracellular organelles. There is evidence, at least in neurons, that IP_3Rs may be directly activated by Gβγ subunit following activation of a G_i protein (297).

Equally important to the release of calcium is the reuptake of calcium into intracellular stores to allow relaxation. The sarcoplasmic reticulum is capable of accumulating millimolar concentrations of calcium and this is returned after release by way of ATP-dependent pumps known as SERCA pumps (sarcoplasmic reticulum, endoplasmic reticulum Ca^{2+} ATPase).The SERCA family of genes encode five isoforms that are all sensitive to the inhibitor thapsigargin (170). Like the plasma membrane Ca^{2+} ATPase and Na^+/K^+ ATPase, SERCA utilize the energy produced from the hydrolysis of ATP to drive the pumping of calcium back into sarcoplasmic reticulum stores. Other means of removing calcium from the cytoplasm and restoring resting intracellular calcium also exist, such as sarcolemmal Na^+/Ca^{2+} exchange and mitochondrial calcium uptake (Fig. 3).

In nonexcitable cells the predominant mechanism for release of store-operated calcium (SOC) is by calcium release activated current (CRAC) channels. The best characterized SOC influx channel is the CRAC channel expressed in T lymphocytes that is essential to the immune response including the regulation of gene expression and cell proliferation. The increase in intracellular calcium occurs in a biphasic manner typically with an initial transient increase due to binding of an agonist such as IP_3 and release of internal stores. The depletion of intracellular calcium stores then triggers release of calcium via CRAC channels on the plasma membrane and is entirely dependent on extracellular calcium. Recent evidence

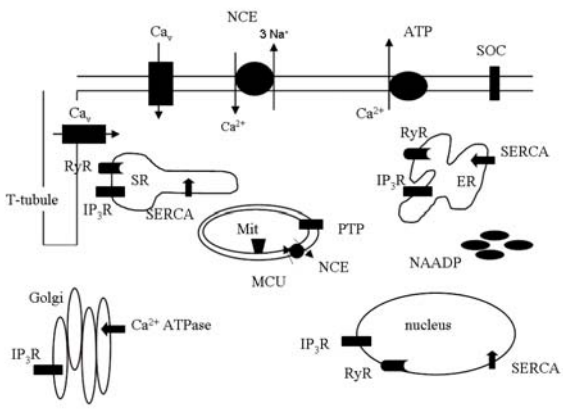

FIG. 3. Channels and transporters responsible for regulating calcium in a cell. ER, endoplasmic reticulum; MCU, mitochondrial calcium uniporter; Mit, mitochondria; NAADP, nicotinic acid adenine dinucleotide phosphate-sensitive calcium channel depicted here on lysosomes; NCE, Na^+/Ca^{2+} exchanger; PTP, mitochondrial permeability transition pore; SERCA, sarcoplasmic endoplasmic reticulum ATPase; SOC, store operated channel; SR, sarcoplasmic reticulum.

indicates that a transmembrane protein with an EF hand motif (a helix-loop-helix Ca^{2+} binding motif) near the amino terminus located in the lumen of the ER termed STIM-1 is the sensor that migrates from the calcium store to the plasma membrane to activate CRAC channels (298). CRAC channels have a low single channel conductance, about 25 fS for calcium and 0.2 pS for monovalent cations. They favor opening when the cell is hyperpolarized (301).

C. Mitochondrial and nuclear ion channels

Many organelles other than the sarcoplasmic reticulum and endoplasmic reticulum are able to take up calcium and impose strict control over its movement to regulate organelle function. As mentioned above, the Golgi complex expresses IP_3Rs. It also contains SERCA-type pumps known as secretory pathway Ca^{2+}-ATPase that are located in close proximity to the nuclear membrane and hence may be involved in calcium signaling with the nucleus (281). Calcium uptake into Golgi may occur by thapsigargin-sensitive and thapsigargin-insensitive mechanisms. Interestingly, only the *cis*-Golgi express IP_3Rs unlike the *trans*-Golgi, suggesting that the role of the *trans*-Golgi in vesicle packaging and post-translational modification has differential requirements for calcium compared with the *cis*-Golgi that appears to play a greater contribution to spatial and temporal calcium signals (268). Calcium release channels that do not involve IP_3 or cADPR have been identified on lysosome-related organelles. A nicotinic acid adenine dinucleotide phosphate (NAADP)-sensitive calcium channel has been located on lysosomes in pancreatic β cells that requires proton exchange to assist with calcium loading (287).

It is well established that the mitochondria can accumulate calcium. Control of mitochondrial calcium is necessary for ATP production (174) and the shaping of intracellular calcium signals (133). Calcium overload is associated with the processes mediating necrosis and apoptosis (programmed cell death) (109). In addition, several of the mitochondrial enzymes require calcium as a cofactor in higher concentration in the mitochondrial matrix than the mitochondrial cytosol, therefore transport of calcium across the mitochondria must be carefully regulated (106). Calcium accumulation mainly occurs through the mitochondrial calcium uniporter (MCU) (see Fig. 3) located on the inner mitochondrial membrane. Calcium transport through MCU requires several hundred nanomolar calcium for activation (half maximal activation of 30 μ*M*) (215, 222). Recently, the MCU was identified as a calcium channel with extremely high calcium affinity (pore binding dissociation constant ≤2 n*M*) and biophysical characteristics that included an inwardly rectifying current suited to calcium uptake into mitochondria and inhibition by ruthenium red (143). Calcium may exit the mitochondria via the permeability transition pore (PTP) that is more frequently associated with the collapse of the membrane potential and influx of proteins that lead to cell death (109, 110). PTP opening is thought to occur when a protein, cyclophilin D, binds to the matrix side of the PTP. Calcium is required for the binding of cyclophilin D. Calcium efflux from the mitochondria also occurs via the Na^+/Ca^{2+} exchanger that, similar to the plasma membrane exchanger, transports one calcium ion out of the mitochondria in exchange for three sodium ions. In addition in some cell types, calcium is extruded from the mitochondria via a Ca^{2+}/H^+ exchanger that can be inhibited by cyanide (105). Mitochondrial calcium uptake is also necessary for production of reactive oxygen species by the mitochondria. This will be discussed in further detail in Section VI.

Although it is recognized that regulation of calcium within the nucleus is vital for many functions including gene transcription, little is understood about the regulatory processes involved. The nuclear membrane consists of an envelope with a luminal space capable of storing calcium. It also possesses the components necessary to regulate calcium levels such as SERCA, IP_3Rs, and RyRs (96). Both IP_3Rs and RyRs are located on inner and outer nuclear membranes and the nucleus can generate its own IP_3. The outer nuclear membrane is continous with the ER and the inner nuclear membrane with the SR such that they regulate the filling and emptying of the nuclear envelope calcium pool (172). There is also evidence that the nucleus contains its own stores (termed nucleoplasmic reticulum) that may physically associate with the mitochondria, suggesting a cooperative role in regulating calcium signals (167).

III. PHYSIOLOGICAL ROLES OF Ca^{2+} CHANNELS

A. Muscle contraction

In the heart, calcium influx across the plasma membrane through L-type Ca^{2+} channels in contractile cells that contain well-developed t-tubules is essential for triggering calcium release from the sarcoplasmic reticulum, a process known as calcium-induced calcium release. This occurs as a result of a close physical association between dihydropyridine receptors (L-type Ca^{2+} channels) and transverse or T-tubules that communicate directly with RyRs on the sarcoplasmic reticulum (Fig. 3). Because the activation kinetics of L-type Ca^{2+} channels in skeletal muscle is 100-fold slower than in cardiac muscle, the process of calcium influx is too slow to initiate contraction. In skeletal muscle, therefore, a depolarization of the plasma membrane is the trigger for release of calcium from SR stores rather than the influx of calcium itself. Therefor, L-type Ca^{2+} channels serve as the voltage sensor used to trigger the release of Ca^{2+} from intracellular store for initiating contraction as calcium influx across the plasma membrane is not a requirement (92). In addition, a hormone-induced calcium release can initiate contraction through release of calcium from internal stores via binding to IP_3Rs.

Cytosolic calcium must be removed to allow for the relaxation of muscle fibers. The majority of cytosolic calcium uptake occurs by the SR Ca^{2+}-ATPase, with the remaining calcium being extruded from the cell either by Na^+/Ca^{2+} exchange or a small amount being taken up by the mitochondria via the MCU. Na^+/Ca^{2+} exchange is voltage dependent and reversible with high intracellular calcium favoring calcium efflux, while a positive membrane potential and high intracellular Na^+ favors Na^+ efflux (60). Under physiological conditions, Na^+/Ca^{2+} exchange works mostly to extrude cal-

cium driven by the intracellular calcium transient. It is also a secondary active transport process driven by the Na^+/K^+ ATPase that extrudes three Na^+ ions in exchange for two K^+ ions while hydrolyzing one molecule of ATP. If the Na^+/K^+ ATPase is partially inhibited, calcium influx through Na^+-Ca^{2+} exchange is increased. This is the basis for the increased force of muscle contractions induced by cardiac glycosides in the heart.

IP_3Rs are the main trigger of calcium release from SR and ER in smooth muscle. Generally, calcium release from IP_3Rs stimulates further calcium release in a cooperative fashion, but with high calcium concentrations inhibiting channel function. This creates the classical oscillatory pattern of calcium release typically seen in innervated smooth muscle such as that resulting in peristalsis in the bowel or agonist mediated calcium release pathways.

RyRs inactivate in one of two ways. They either do not reopen until they recover (223, 227), or they relax to a lower open probability but can still be reactivated at high calcium levels, a process known as adaptation and also common to IP_3Rs (107). It was originally proposed that adaptation of RyRs enabled a graded calcium-induced calcium release for contraction in muscle. However, it has been argued that the adaptation response reported by Györke and Fill (107) that was elicited by flash photolysis experiments is inconsistent with a number of recent studies and cannot explain rapid changes in calcium that occur *in vivo* (151, 223, 231).

B. Neurotransmitter secretion

Another important physiological process involving calcium is neurotransmission at nerve terminals. Within the presynaptic terminal of neurons, hormones, neurotransmitters, or other peptides are stored in vesicles. When an action potential depolarizes the membrane, the vesicles fuse with the plasma membrane and the contents are released into the extracellular space where they can bind with receptors and initiate chemical activity in nearby cells. The process of neurotransmission is calcium-dependent in that secretion requires extracellular calcium to enter the presynaptic cell and is inhibited by extracellular Mg^{2+} (65). Intracellular calcium influences the inactivation rate and inhibition of L-type Ca^{2+} channels and activates large conductance K^+ channels (BK(Ca)) that terminate neurotransmitter release in the presynaptic terminal by hyperpolarizing the membrane (217, 238). Calcium-dependent chloride channels also contribute to membrane potential hyperpolarization along with the BK(Ca) channels in neurons (173).

The release of neurotransmitters is steeply calcium dependent, and a decrease in calcium channel activity contributes to presynaptic inhibition (70). An example of presynaptic inhibition is the block of transmission of sensory fibres that occurs with opiates such as morphine. This occurs as a result of rapid coupling of G protein $\beta\gamma$ subunits to Ca_v2 family of calcium channels, causing a slowing of activation of the channels (62). Several modulatory transmitters such as acetylcholine, norepinephrine, adenosine, and GABA contribute to depression of calcium channels at presynaptic terminals through the G protein-dependent mechanism.

Both presynaptic and postsynaptic calcium stores are involved in long-term depression in the developing hippocampus (43). However, presynaptic RyRs appear to be important in determining the strength of synaptic transmission induced by *N*-methyl-D-aspartate (NMDA) receptors (262). Activation of presynaptic NMDA, kainate, and nicotinic acetylcholine receptors can lead to calcium-induced calcium release from RyR stores similar to that reported in heart muscle that acts to inhibit or regulate secretion. Although still controversial, calcineurin may interact with IP_3Rs and RyRs to modulate intracellular calcium release and synaptic plasticity (38).

Calcium can also play a role in building synaptic connections. Rapid changes in synaptic strength are mediated by posttranslational modifications of pre-existing protein, but enduring changes are dependent on gene expression and the synthesis of a variety of proteins required for synaptic transmission. One of the key mediators of gene expression involved in synaptic plasticity is the transcription factor cAMP response element binding protein (CREB). This transcription factor plays an important role in behavioral adaptations to changes in the environment and complex processes such as learning and memory. Both short- and long-term memory are believed to be a result of a process known as long-term potentiation. The mechanism of long-term potentiation involves phosphorylation and activation of NMDA glutamate receptors by Ca^{2+}/calmodulin-dependent protein kinases (CaM kinases) II and IV and MAP kinase. This results in an increase of Ca^{2+} entry into postsynaptic neurons. In addition CaM kinase IV and MAPK increase phosphorylation of CREB and expression of c-Fos (181). Phosphorylation of CREB is also important in proliferation and differentiation in developing vertebrates. Calcium influx through L-type Ca^{2+} channels or NMDA receptors is required for phosphorylation and activation of CREB (146). Another transcription factor responsible for shaping long-term changes in neurons is the NFAT (nuclear factor of activated T cell) family, and similar to regulation of CREB, activation of NFAT is dependent upon calcium influx through L-type Ca^{2+} channels. Interactions of calcium with PDZ domain proteins (PSD-95, Discs-large, ZO-1 domain proteins that often function as scaffolding proteins and have been shown to play important roles in signal transduction) appear to be necessary for the coupling of L-type Ca^{2+} channels to NFAT as does CREB activation (276). Neurotrophins, such as the brain-derived neurotrophic factor, have also been implicated in the activation of NFAT-dependent transcription. Initiating such transcriptions leads to further increase in brain derived neurotrophic factor mRNA and protein as positive feedback (104).

C. Excitability and the action potential

Calcium plays an important role in shaping the action potential of muscle and neurons. One example where the shape and duration of the action potential must be carefully controlled is in cardiac pacemaker activity. The spontaneous activity of pacemaker cells is attributed to a phase of the atrial action potential known as the slow diastolic depolarization (phase 4; Fig. 4). During this phase, the membrane potential slowly depolarizes following termination of an action poten-

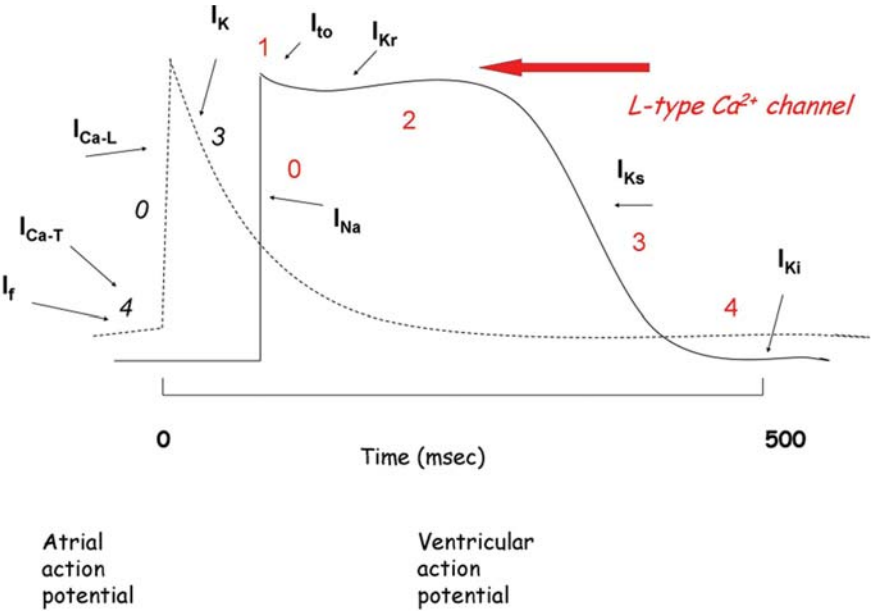

FIG. 4. Action potentials in the heart. The atrial action potential is shown at *left* (*dashed lines*) and ventricular action potential at *right*. Names of the classes of channels that contribute to each phase are also shown. The three phases of the atrial action potential are indicated in *italics*. I_f, hyperpolarization activated or "funny" channel; I_{Ca-T}, T type calcium channel; I_{Ca-L}, L type Ca^{2+} channel; I_K, delayed rectifier K^+ channel; I_{Na}, fast inward sodium channel; I_{to}, transient outward K^+ channel; I_{Kr}, rapid component of delayed rectifier K^+ channel; I_{Ks}, slow component of delayed rectifier K^+ channe; I_{Ki}, inward rectifying K^+ channel (for more detail, see text and Ref. 189). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars.)

tial until the threshold for a new action potential is reached. It is well accepted that the electrical activity of cardiac pacemakers is thought to depend exclusively on voltage-dependent ion channels within the plasma membrane of nodal cells. These include the hyperpolarization-activated inward current (I_f), the time-dependent decay of K^+ conductance, inward L-type and T-type Ca^{2+} currents, and the lack of background K^+ conductance. The hyperpolarization-activated channel carries Na^+ and K^+ ions inwards and activates upon hyperpolarization. However, interventions that affect intracellular Ca^{2+} can also affect pacemaker activity (158). A rise in intracellular Ca^{2+} results in activation of the Na^+/Ca^{2+} exchanger. This produces an inward current that is sufficient to induce diastolic depolarization and pacemaker activity (134). Activation of T-type Ca^{2+} channels has been implicated in the increase in intracellular Ca^{2+} (126). The importance of Ca^{2+} in contributing to pacemaker firing is further supported by studies that show that embryonic stem cell-derived cardiomyocytes do not show increased beating rate with differentiation when they lack ryanodine receptors (289).

The rate of change in membrane potential during the upstroke is much slower in sino-atrial node cells than in ventricular cells. This is because depolarization of the membrane during the rapid upstroke (phase 0) of a slow response action potential in pacemaker cells is caused by an increase in the Ca^{2+} conductance due to activation of L-type Ca^{2+} channels. Nodal cells have fewer Na^+ channels than ventricular cells and because the membrane potential in phase 0 is depolarized, changes in Na^+ conductance do not contribute. Pacemaker cells do not have a pronounced plateau phase. Action potential duration is determined by a balance between Ca^{2+} and K^+ conductances.

In ventricular myocytes the prolonged phase 2 (plateau phase) of the action potential is characterized by slow inward current produced by L-type Ca^{2+} channels (see Fig. 4). This is a distinguishing feature of the cardiac action potential and is the necessary trigger for calcium-induced calcium release and contraction. Similarly, in smooth muscle and skeletal muscle, calcium influx through L-type Ca^{2+} channels is important in shaping the duration of the action potential; however, the duration is much shorter than that of ventricular myocytes. In smooth muscle and neurons, the calcium-dependent large conductance K^+ channels contribute to hyperpolarization of the membrane.

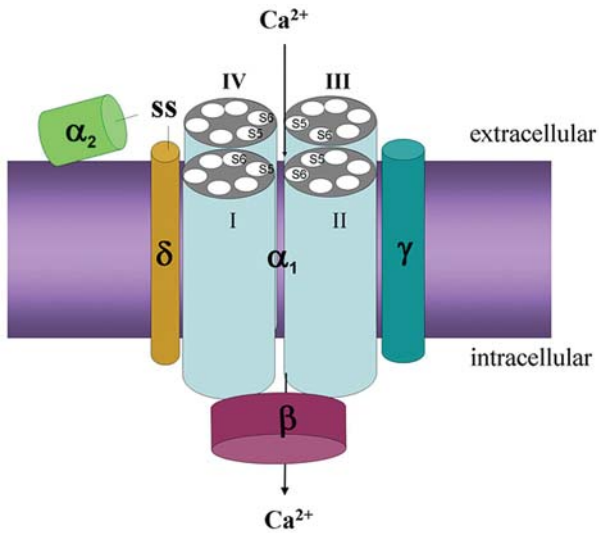

FIG. 5. The likely structure and arrangement of the L-type channel subunits. The pore is formed by the S5 and S6 helices and linker of the α1 subunit. The β subunit interacts with the linker between domains I and II of α1 and modifies gating and inactivation kinetics. The δ and γ subunits lie in the membrane, while α2 is accessible to the extracellular solution. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars.)

IV. STRUCTURE OF VOLTAGE-GATED CHANNELS

Although no atomic resolution structure has been determined for any calcium channels, we still know a great deal about the molecular structure and function of the voltage-gated family. The reason for this is that these channels are closely related to the voltage-gated sodium and potassium channels and much that is learnt about one of these classes can be transferred to the others. In particular, a great deal has been learned about the structure of voltage-gated calcium channels since the recent determination of crystal structures of a number of potassium channels (66, 130, 162).

A. Subunit structure and function

Voltage-gated calcium channels are formed by the association of separate subunits, that come together to form an active channel. The variety of voltage-gated calcium channels arises from differences in the sequences of the individual subunits comprising the channel, in particular the α1 subunit. As with voltage-gated sodium channels, but unlike the potassium channels, the pore forming domain of the calcium channel protein is comprised of four regions of the one subunit rather than by separate subunits.

Calcium channels were first purified from transverse tubule membranes of skeletal muscle (57) where it was found that the channel was comprised of five subunits: α1 weighing 190 kDa and later shown to form the pore; a disulfide linked α2-δ dimer of 170 kDa; and an intracellular phosphorylated β subunit weighing 55 kDa; and a transmembrane γ subunit of 33 kDa as indicated in Fig. 5 (122, 154, 239, 246).

The α1 subunit has a structure similar to the previously cloned pore forming subunit of the voltage-gated-Na^+ channels. It contains four repeated domains (I to IV) each of which includes six transmembrane segments (S1 to S6) and a membrane associated loop (the 'P-loop') between segments S5 and S6 as indicated in Figs. 1 and 5 (1). That the α1 subunit forms the ion conducting pore is supported by the fact that expression of just this subunit is enough to produce functional channels, albeit with unusual kinetics and voltage dependence (202). Each of the six transmembrane segments most likely form α-helices. The four repeated domains surround the ion conducting pore, with the S5, S6, and P-loops lining the conduit. Much evidence and analogy with the potassium channels implies that ion selectivity takes place in a region surrounded by the four P-loops. Replacement of particular lysine or alanine residues in the P-loop of a voltage-gated sodium channel with glutamate residues changes the channel from being sodium selective to calcium selective (115). Not only does this suggest that this region of the pore is critical for ion selectivity, the fact that all calcium channels have conserved glutamate residues at this position suggests that it is these glutamates that are crucial for Ca^{2+} selectivity. Further site-directed mutagenesis indeed determined that four glutamate residues, one from each of the P-loop of the channel (often called the 'EEEE locus'), were responsible for the high affinity calcium binding site. Mutation of one or more of these had a significant effect on the channel selectivity (17, 73, 142, 200, 290).

The opening of calcium channels does not happen instantaneously after depolarization, but rather after a delay of one or more milli-seconds depending on the channel type and membrane potential as illustrated in Fig. 6. This is generally taken to mean that the channel protein must move through many closed conformations before channel opening occurs. The S4 helix contains a number of arginine residues and is believed to act as the voltage sensor (7, 93). By similarity with K^+ channels, it is expected that the channel gate lies at the internal end of the S6 helices, which most likely form a narrowing of the pore in the closed state.

B. Redox sensitive sites

Cysteine residues are generally thought to be the most likely target of redox or nitrosylation modification in proteins, as free thiols can easily react with oxygen or reactive nitrogen species and can be assisted in forming intramolecular disulfide bonds (45, 97, 159). For this reason the location of the cysteine residues is important when considering the mechanism of redox regulation of calcium channels. Voltage gated calcium channels contain many cysteines. The α_1 subunit from L-type channels, for example, has 48 (Fig. 7) Not all of these will be susceptible to oxidation or reactions, however, as many will already be involved in disulfide bonds. As described later in Section VIII, oxygen could influence ion channels through many pathways, but it has been shown that Ca^{2+} currents are altered by thiol reducing and oxidizing agents (54, 83, 118, 119, 150, 188, 255), demonstrating that free thiols in the protein are likely to be sensitive to the oxidation state of the cell, and furthermore that these cysteines may be sensitive to hypoxic conditions (82, 85, 118, 119). The region of the L-type channel responsible for this sensitivity was also deduced by Fearon and colleagues who demonstrated that one out of three naturally occurring splice variants, differing only in 71 amino acids near the C-terminal tail, were sensitive to oxygen in this way. Removal of a further 24 residues localized the oxygen sensitive region to a 39 amino acid region (Fig. 7) (85). The evidence for an involvement of cysteines in hypoxic responses is discussed further in Section VIII.

Another target for redox regulation of channel function is methionines. Oxidation of methionines within the pore of large conductance Ca^{2+}-activated K^+ channel causes an increase in channel activity (221, 250). Interestingly, thiol-specific oxidizing agents inhibit channel function, suggesting

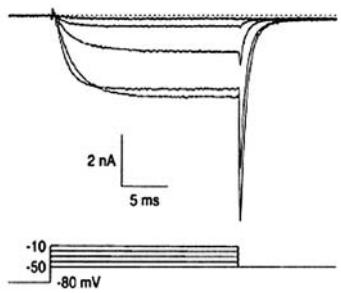

FIG. 6. Activation kinetics of whole cell inward calcium currents in frog sympathetic neurons. Measurements are made with 2 m*M* Ba^{2+}, and the largest currents are at −20 mV and the largest tail currents at −50 mV. Figure reproduced with permission from Ref. 131a.

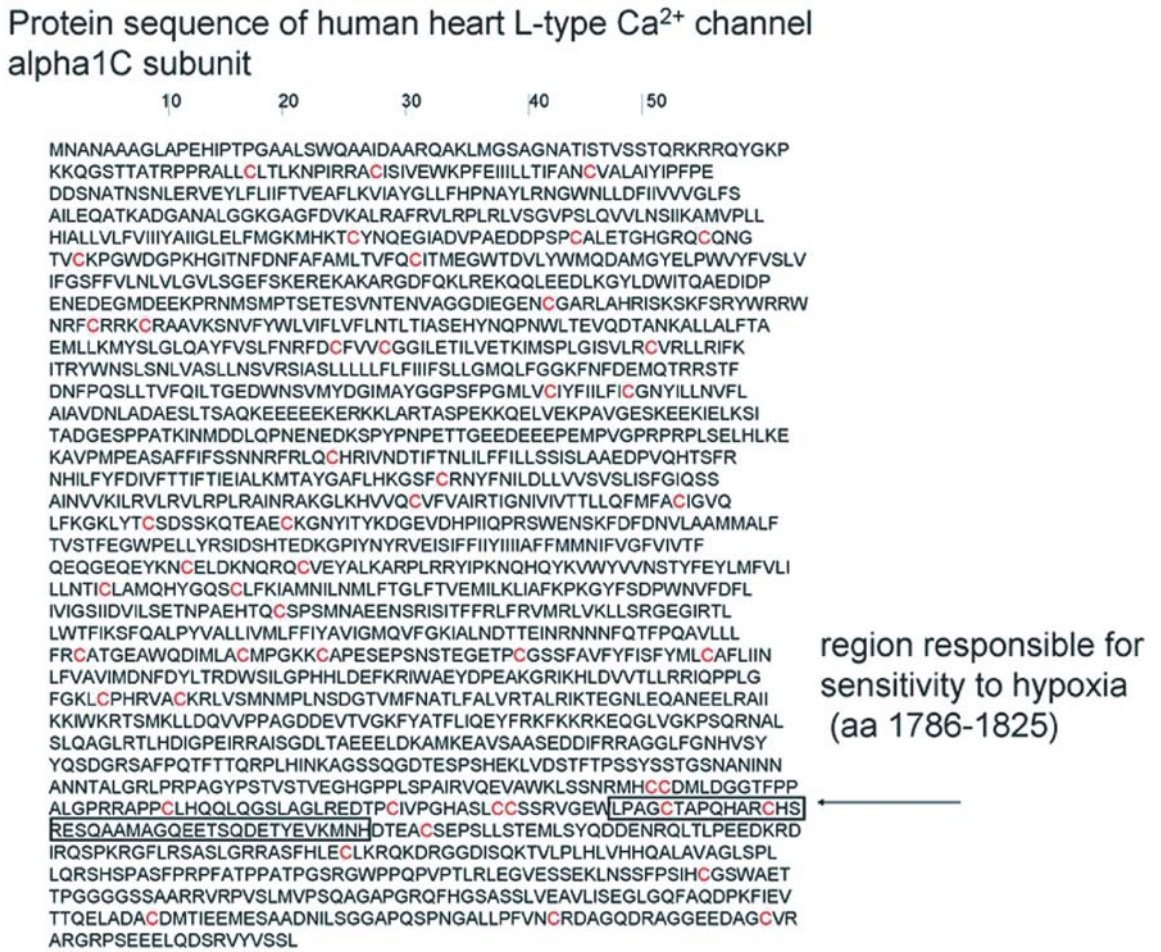

FIG. 7. The protein sequence of alpha 1C subunit of the human L-type Ca^{2+} channel, with cysteines indicated in *red*. The region responsible for conferring sensitivity to hypoxia is *boxed* and indicated at *right* (see Ref. 85, and text for more detail). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars.)

that variation in responses may be due to whether methionines or cysteines are targeted and reactive. Shaker type K^+ channels exhibit more rapid P/C-type inactivation due to oxidation of methionines on the channel protein (50). Oxidation of methionines on calmoldulin increases interaction of calmoldulin with RyR1 (19). Therefore, the oxidation of regulatory proteins such as calmodulin may play a more important role in altering channel function than oxidation of the channel protein itself. The role of methionines in redox regulation of many of the ion channels and transporters has yet to be determined.

V. STRUCTURE OF CALCIUM RELEASE CHANNELS

In comparison to voltage-gated channels, surprisingly little is known about the structure of ryanodine and IP_3 receptors, in most part due to the lack of an atomic resolution picture of these or related proteins. One of the reasons for this is the enormous size of these proteins that makes crystalization and characterization difficult. RyRs, for example, are made from subunits containing over 5,000 amino acid residues, while IP_3Rs are made from subunits of over 2,700 residues. In both cases, the functional channel is formed as a homotetramer of four subunits, which for the RyRs makes a total molecular mass of ~2.3 MDa. Determining the location and function of each portion of these proteins remains an ongoing project.

All three RyR isoforms show strong sequence homology, with over 66% identity between each pair. Conversely, there are specific regions that show a large degree of sequence diversity (residues 1302–1406, 1864–1925, and 4250–4627) that may be responsible for the functional differences of the isoforms (69). The most detailed structural information comes form an 14 Å resolution picture of RyR1 from cryo-electronmicroscopy (226). The structure is described as a 'square mushroom' with a bulky cytoplasmic domain attached to a smaller transmembrane stem. The cytoplasmic domain is over 270 Å across and extends over 100 Å from the membrane. The four subunits of the protein join in a roughly square shape with the large cytoplasmic domain residing between the SR and T-tubule membranes. In skeletal muscle, the dihydropyridine receptors in the plasma membrane are

arranged in regular arrays with the RyRs, with four dihydropyridine receptors facing every second RyR, where they are probably in physical contact with the large RyR cytoplasmic domains. Deletion of all but the C-terminal 1000 residues leaves a functional channel that binds ryanodine, is activated by Ca^{2+}, and retains wild-type conductance (256). Thus, there is considerable evidence that the ion conducting pathway is formed by this part of the protein.

IP_3Rs have many structural similarities to the RyRs. Again the transmembrane domain is believed to be largely comprised of the C-terminal region of the protein that has relatively high sequence homology with the similar region in RyRs. The N-terminal 1,500 residues, on the other hand, show very little similarity to the RyRs. In type I IP_3Rs, the Ca^{2+}-binding sites lie within the inositol triphosphate (IP_3)-binding core, and it has been proposed that calcium binding is negatively regulated by a conformational constraint imposed by IP_3-binding (33). A region with similar sequence to this binding core is also present in the ryanodine receptors, suggesting some commonality.

Each of the homologous subunits in the RyRs contains about 100 cysteine residues. Therefore, there is plenty of scope for redox or nitrosylation modification (249). Of these, about 50 appear to be in the reduced state, and thus available for reactions, as deduced from reacting small fluorescent thiol-reactive molecules with the protein. In the native RyR1, about 7–12 are highly susceptible to oxidation by exogenous sulfhydryl reagents (77, 243). Furthermore, the location of seven of these hyperreactive sites within the primary sequence has been identified using mass spectrometry (270). In addition to Cys-3635 that was previously known to react with NO, cysteines at positions 1040, 1303, 2436, 2565, 2606, and 2611 have been shown to react with fluorescent sulfhydryl probes. IP_3Rs also appear to be susceptible to redox modification, with about 70% of the 60 cysteines in the type 1 IP_3R maintained in the reduced state. However, only a few of these appear to reside on the surface of the protein, as only a few residues in the ligand binding and central coupling domain of the receptor are accessible to bulky reagents (132).

VI. REACTIVE OXYGEN SPECIES AS SIGNALS

A. Role of reactive oxygen species in cell function

Oxygen is the substrate for the generation of reactive species that play an important role in modulating cardiac cell function, and when produced at high levels induce cell death. Oxidative stress caused by reactive oxygen species (ROS), also described as free radicals, is usually considered to be directly detrimental to the health of organisms because ROS can damage key macromolecules such as DNA, proteins, and lipids (111). However, there is now compelling evidence that ROS can also act as signaling molecules able to stimulate and modulate a variety of biochemical and genetic systems. ROS have been shown to affect the regulation of signal transduction pathways, gene expression, cytokine production, differentiation, proliferation, senescence, and cell death by apoptosis (140, 216, 245, 253).

The term reactive oxygen species is a generalized description for a number of reactive oxygen molecules of biological significance (112). ROS are generally speaking oxygen molecules in different states of oxidation or reduction, as well as compounds of oxygen with hydrogen and nitrogen. Although superoxide is produced directly from the reduction of oxygen, the biologically active species are hydrogen peroxide, hydroxyl radicals, hypochlorite ion, and peroxynitrites (Fig. 8). Superoxide is produced as a result of the donation of an electron to oxygen. It is a weak base at physiological pH, highly soluble in water and therefore does not easily cross lipid membranes, although it can be transported via anion channels (199, 266). It is also a one-electron reductant of metals. Superoxide can also be protonated to hydroperoxy radical at low pH ($pK_a = 4.8$), but the principal fate of superoxide at physiological pH lies in its dismutation to hydrogen peroxide by superoxide dismutase. The rate of dismutation is rapid ($k = 1.6 \times 10^9\ M^{-1}s^{-1}$) maintaining the concentration of superoxide in the picomolar-nanomolar range (25). Since superoxide does not easily cross lipid membranes, it has been argued that

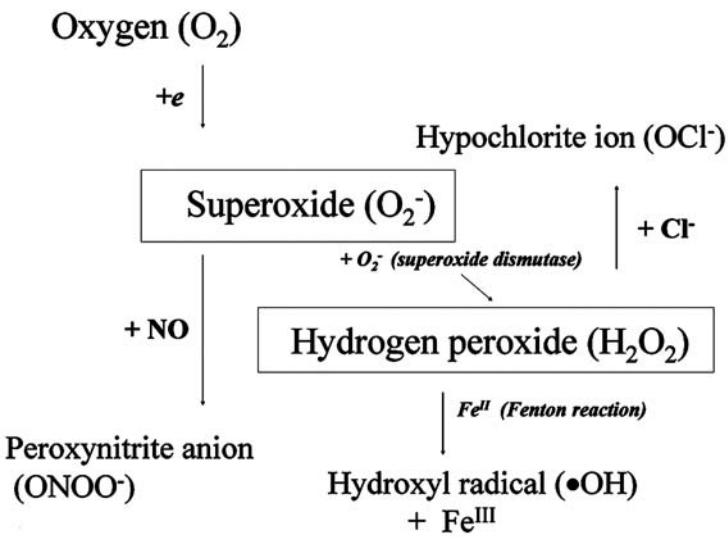

FIG. 8. Schematic showing the generation of reactive species arising from molecular oxygen (O_2). Reactions with chloride ion (Cl^-), nitric oxide (NO), and iron (Fe^{II}) are as indicated. +e, addition of an electron; O_2^-, superoxide anion.

its role may be limited to oxidation of proteins in the organelle in which it is produced (253).

In contrast to superoxide, hydrogen peroxide is a highly stable small molecule that freely crosses membranes as its biological diffusion properties are similar to water (11). This makes hydrogen peroxide a biologically important candidate as a ROS and signaling molecule. The naturally occurring enzyme (and antioxidant) catalase metabolizes hydrogen peroxide to water and oxygen and is principally responsible for maintaining intracellular hydrogen peroxide in the nanomolar range (11, 196, 237). Hydrogen peroxide can participate in one-electron reactions with metal ions (Fenton reaction) and generate hydroxyl radicals. A hypochlorite ion is generated when hydrogen peroxide reacts with Cl^-. Both hydroxyl radical and hypochlorite ion are highly reactive (23, 280). Other reactive species are derived from nitric oxide, a well-recognized signaling molecule implicated in cardiovascular function as an important vasodilator. Nitric oxide reacts with oxygen to produce nitrogen dioxide that participates in the peroxidation of lipids and with superoxide to produce peroxynitrite, also a stable strong oxidant of lipids implicated in vascular disease (185, 279).

Hydrogen peroxide is believed to interact with cell signaling pathways by way of modification of key thiol groups on proteins that possess regulatory functions. These proteins may be second messengers such as serine/threonine, tyrosine, and MAP kinases, growth factors, and transcription factors such as NF-κB (253). Hydrogen peroxide precisely regulates the catalytic activity of enzymes by redox modification of cysteine residues (20). These include tyrosine phosphatase activity (220, 265), the translocation and activation of serine/threonine kinases such as protein kinase C (248), and the induction of gene expression (20).

Under certain circumstances an excess of ROS is beneficial to the host when defending against microorganisms or pathogens in plants (197). Although ROS may be required for cell signaling and normal cellular function, excessive production of ROS is deleterious to the mammalian cell. ROS have been implicated in a variety of pathophysiological conditions including senescence and aging, ischemia–reperfusion injury, atherosclerosis, hypertension, and diabetes (see section below). Therefore, understanding the role that ROS play in mediating pathology provides insight into mechanisms of disease and ways to abrogate the progression of disease.

B. Sites of production of reactive oxygen species

A number of sites within the cell are known to participate in the production of ROS. Some organelles such as the endoplasmic reticulum and peroxisomes in the liver and kidney produce hydrogen peroxide and superoxide, but because most of the ROS do not cross the membranes of the organelles, their roles are limited to local oxidation of proteins (253). Xanthine oxidase utilizes hypoxanthine, xanthine, and NADH to form superoxide and H_2O_2 in cardiac myocytes and vascular smooth muscle cells (171). The ratio of xanthine oxidase to xanthine dehydrogenase can be used as an indicator of oxidative stress in a cell. Recently it was established that xanthine oxidase is a physiological regulator of cyclooxygenase 2 expression, further establishing a link between inflammatory responses and vascular cell injury in cardiovascular disease (192).

A significant source of ROS in vascular smooth muscle, phagocytes, endothelial cells, and cortical neurons is the plasma membrane-bound nicotinamide adenine dinucleotide phosphate (NAD(P)H)-oxidase. This complex is a plasma membrane-associated enzyme that reduces O_2 to superoxide with the transfer of one electron from NADH or NAD(P)H. Superoxide is then converted to H_2O_2 by superoxide dismutase. The orientation and subunit composition of the enzyme appears to vary in different tissues (40). The phagocytic oxidase appears to produce large amounts of superoxide on the external surface of the plasma membrane as defense against inflammatory cells. The vascular oxidases appear to generate a low basal level of superoxide in the absence of cellular stimuli although a number of stimuli increase the activity and expression of the oxidase (102). The NAD(P)H oxidase complex is composed of the electron transfer components (gp91phox and p22phox) and the regulatory p67phox and p47phox subunits. The low molecular weight G protein rac also regulates function of the enzyme. In vascular cells it has been implicated in the mechanisms mediating hypertension and cell growth and inflammation associated with atherosclerosis (102). NAD(P)H oxidases are also regulated by growth factors and cytokines. Angiotensin II is a potent regulator of oxidase function in vascular smooth muscle and the heart (209, 293). In addition, physical factors such as stretch and shear stress increase ROS production via activation of the oxidase (40).

Another source of production of superoxide is the NADH oxidase decribed in skeletal muscle and the heart. The NADH oxidase is closely associated with the sarcoplasmic reticulum and its activity is coupled to activation of calcium release from SR in skeletal muscle through type I RyR (283). In cardiac myocytes, however, NADH inhibits RyR2 mediated calcium-induced calcium release and significantly decreases RyR2 open probability (53). Although the molecular identity of this oxidase has not yet been determined, the oxidase appears to be functionally distinct from the plasma membrane-associated NAD(P)H oxidase. Photoaffinity labeling with [^{3}H](trifluoromethyl)diazirinyl-pyridaben identified a single 23 kDa PSST-like protein in cardiac junctional SR . NADH ubiquinone oxidoreductase in mammalian mitochondria possesses a nuclear-encoded PSST subunit with a binding site for pyridaben. The PSST-like protein appears to co-localize with NADH-oxidase in SR of RyR2 complex in the heart but not RyR1 in skeletal muscle (53).

A major source of production of ROS in mammalian cells are the mitochondria. This appears to be the price that eukaryotes pay for oxygen-dependent metabolism. Electron transport within the mitochondria is generally very efficient. However, a small leakage of single electrons can participate in the reduction of oxygen to superoxide. Most of the generation of superoxide and some hydroperoxy radical occurs at the ubisemiquinone site in complex III, but complex I may also be a source of ROS when electrons flow backwards from complex II (259, 260). In addition, superoxide production has been shown to be dependent on mitochondrial membrane potential (236). Generally speaking, ROS production is maximal when the electrochemical potential due to the gradient

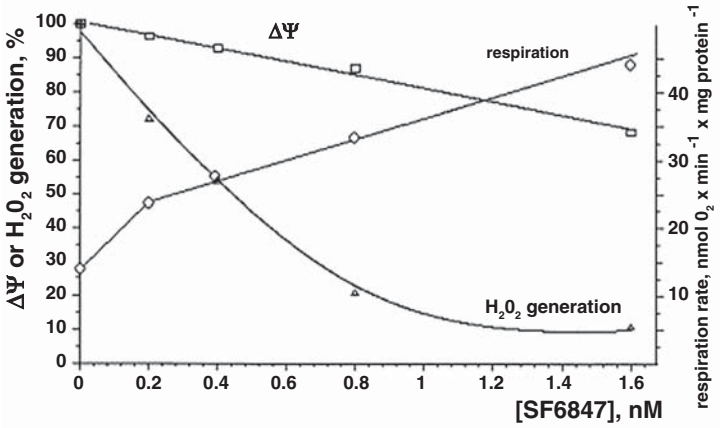

FIG. 9. Effects of SF6847 (a mitochondrial membrane potential uncoupler) on respiration, mitochondrial membrane potential (ΔΨ), and H_2O_2 production by rat heart mitochondria. SF6847 caused a small decrease in membrane potential (about 13%), a 2.2-fold increase in respiration rate, and about 80% inhibition of H_2O_2 production. Incubation mixture: 250 m*M* sucrose, 1 m*M* EGTA, 10 m*M* KCl, 10 m*M* MOPS (pH 7.3), 6 m*M* succinate, rat heart mitochondria (0.8 mg protein/ml), 16 μ*M* safranin O, 1.6 μ*M* scopoletin, horseradish peroxidase (10 U). For H_2O_2 production measurements, safranin O was omitted, and mitochondrial protein was 1 mg/ml. H_2O_2 production and ΔΨ levels in samples without SF6846 were taken as 100%. Reproduced with permission from Ref. 147.

of protons across the inner mitochondrial membrane is maximal. A small decrease in the protonic potential causes a significant decrease in ROS production without affecting energy production (Fig. 9) (147, 236). Partially uncoupling the mitochondrial membrane potential with the addition of 2 n*M* carbonyl cyanide *p*-(triflurometho xy)phenyl-hydrazone (FCCP) to adult guinea pig ventricular myocytes produces a 31% decrease in the rate of dihydroethidium (DHE) signal (121). Oligomycin, an inhibitor of ATP synthase, alone does not affect superoxide production but addition of 0.5 n*M* FCCP causes a 68% decrease in DHE signal. This is good evidence that mitochondria are a significant source of ROS, and ROS production is dependent upon mitochondrial membrane potential in the absence of ATP depletion. ROS are also produced as a result of the $NAD(P)^+$-linked substrate dehydrogenases in the mitochondrial matrix and the activity of the matrix pyridine nucleotides such as monoamine oxidase (10).

An important determinant of cellular redox state is the cell's antioxidant defense (Fig. 10). As mentioned above, the principal fate of superoxide lies in its rapid dismutation at physiological pH to hydrogen peroxide by superoxide dismutase. In vascular tissue a copper–zinc form of superoxide dismutase is predominantly expressed and active in the cytosol, whereas mitochondria possess a manganese form of the enzyme. The presence of these forms of superoxide dismutase ensure that intracellular superoxide concentrations are maintained within physiological concentrations (280). The naturally occurring enzyme (and antioxidant) catalase metabolizes hydrogen peroxide to water and oxygen (196). Hydrogen peroxide can also be converted to water and oxygen by glutathione peroxidase. The ratio of reduced to oxidized glutathione is often used as a biochemical marker of cellular oxidative stress. Less than 5–10% of total cellular glutathione should normally be in the oxidized form (111). Reduced and oxidized glutathione can be assayed spectrophotometrically using the fluorimetric probe *o*-phthalaldehyde (225).

VII. EVIDENCE FOR REDOX REGULATION OF CALCIUM CHANNELS AND TRANSPORTERS

Having discussed the importance of calcium channels in cellular function and indicated that ROS can modulate cell function, we next highlight the evidence that ROS can directly regulate the function of calcium channels and transporters.

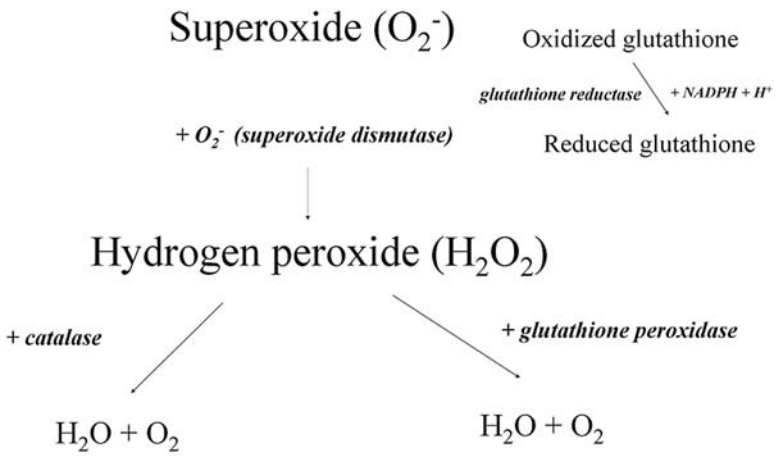

FIG. 10. Antioxidant defense in cells. Superoxide dismutase rapidly dismutates superoxide to hydrogen peroxide, and hydrogen peroxide is broken down to water and oxygen by catalase or glutathione peroxidase. Oxidized glutathione is converted to reduced glutathione by glutathione reductase (see text for more detail).

A. L-type Ca^{2+} channels

Redox regulation of calcium channels is believed to involve an alteration in the reduction/oxidation state of the channel that results in a functional change in the protein. In the native cell, the target may be the channel protein itself or a regulatory intermediate (such as a G protein or serine-threonine/tyrosine kinase). To determine whether cysteines on the channel protein may be affected by changes in redox state, thiol-specific reducing or oxidizing compounds have been applied to the channel and changes in channel activity recorded. In guinea pig ventricular myocytes, the thiol-reducing agent dithiothreitol decreases basal native L-type Ca^{2+} current while 5,5′-dithio-bis[2-nitrobenzoic acid] (DTNB), a thiol-specific oxidizing agent increases basal current (119). Similarly in frog ventricular myocytes, thiol oxidizing agents increase L-type Ca^{2+} current (286). The effect can be prevented by dithiothreitol but is unaffected by an inhibitor of cAMP or application of GDPβS indicating that the response is independent of cAMP production or G protein stimulation. In contrast, the mercury compound *p*-hydroxy-mercuric-phenylsulphonic acid (PHMPS) has been reported to decrease basal L-type Ca^{2+} current (150). In cloned rabbit smooth muscle L-type Ca^{2+} currents are inhibited by 2,2′-dithiodipyridine (DTDP), a specific lipophilic oxidizer of sulfhydryl groups and dithiothreitol reverses this effect (54). Dithiothreitol has no effect on human cardiac L-type Ca^{2+} channels expressed in HEK 293 cells but reverses the inhibitory effects of another oxidizing agent thimerosal (83). It is evident that thiol reducing or oxidizing compounds can alter channel function but the response appears to vary depending on the cell type. These contradictory responses may be due to a contribution by the auxiliary subunits of the native channel present in native cells but absent in the expressed (cloned) channel in which only the α subunit is present. For Ca^{2+}-dependent K^+ channels, there is evidence to suggest that the auxiliary β_3 subunit participates in movement of ions through the α subunit by forming gates that block pore access (297). This gating mechanism is abolished by reduction of extracellular disulfide linkages. Therefore, auxiliary subunits may play important regulatory roles where thiol groups are modified by redox agents. Alternatively, the activity of a regulatory protein such as protein kinase A that can be altered by hydrogen peroxide, thiol-specific reducing or oxidizing agents may then influence channel function (35, 124, 125).

How does thiol modification translate into physiological or pathologically relevant alteration in ion channel function? In the case of the native L-type Ca^{2+} channel, dithiothreitol mimics the effects of acute hypoxia (PO_2 17 mm Hg) and DTNB attenuates the hypoxic inhibition of basal channel activity (119). This allows some insight into how acute hypoxia influences L-type Ca^{2+} channel function and the mechanisms for the triggering of calcium-mediated arrhythmias associated with decreased oxygen delivery to cardiac myocytes. In the case of sensory neurons, thiol-reducing agents induce thermal hyperalgesia via increased T-type ($Ca_v3.2$) channel activity but do not affect NMDA receptors or voltage-dependent Na^+ or K^+ channels (188, 255). This may occur as a result of direct redox modification of the channel as recombinant $Ca_v3.2$ channel activity was also increased by dithiothreitol. However, in rat cortical neurons oxidized glutathione inhibits NMDA receptor activity via an effect on a redox modulatory extracellular site (241), suggesting that peripheral and central neurons are regulated differentially by redox state. Oxidation by hydrogen peroxide enhances L-type and P/Q type calcium channel activity (120, 123, 155). The enhanced calcium influx suggests oxidation of the channels may play an important role in the oxidative stress response. Membrane lipid peroxidation induced by application of 4-hydroxy-2,3-nonenal increases neuronal cell death as a result of enhanced calcium influx through NMDA channels (166). Although the source of calcium may be debated, these data emphasize the relationship between calcium influx through calcium channels and the role of calcium in cell viability during oxidative stress.

A number of contradictory observations have been reported regarding the role of nitric oxide in the regulation of Ca^{2+} channels. Nitric oxide is generated from L-arginine by one of the three isoforms of nitric oxide synthase (endothelial, neuronal, or inducible NOS). In ferret heart, nitric oxide can regulate L-type Ca^{2+} channels either through direct redox-dependent activation or indirectly via cGMP-dependent inhibition (45). Nitric oxide augments P/Q type channels ($Ca_v2.1$) and dithiothreitol attenuates the effect, suggesting that nitric oxide or a reactive nitrogen species may be acting directly to alter channel function in neurons (51). However, in rat insulinoma cells, P/Q type and L-type Ca^{2+} channels are inhibited by nitric oxide (101). It is now recognized that the divergent views may be a result of spatial localization of nitric oxide synthases, indirect effects of secondary pathways such as cGMP-dependent protein kinase, and the role of reactive nitrogen species (21, 100, 141).

B. Ryanodine receptors and IP_3 receptors

There is good evidence to suggest that the redox state of cysteines on the RyR is responsible for altered channel function. The RyR is a tetramer approximately 2.3 MDa in size. Each monomer contains approximately 100 thiols. Although most of these will not be free for covalent modification as they may be already bound to proteins through disulfide links or buried within the structure of the receptor, it is estimated that up to half are free thiols (68, 243). Early studies showed binding of heavy metals or thiol oxidizing compounds caused calcium release from SR and increased contraction in skeletal muscle as a result of oxidation of critical thiol groups on the calcium release channel (2, 257, 295). Hydroxyl radical also activates calcium release through RyRs (12). The reactive thiols can respond differentially (inhibit or activate the channel) depending on whether the residues are located on the luminal or cytoplasmic side of the protein (4, 72). In addition, thiol oxidizing agents have reported to sensitize the RyR to activation by calcium while desensitizing inhibition by magnesium (63, 116). Exposing the channel to increased levels of reduced glutathione decreases the binding affinity of ryanodine for its receptor (292). The cardiac RyR appears to be very sensitive to nitrosylation and is nitrosylated *in vivo*. *S*-nitrosylation of up to 12 thiols on the tetramer results in channel activation but oxidation of thiols does not influence channel function unless additional thiols are oxidized that then leads to loss of control (irreversible activation) (78, 285). Molecu-

lar oxygen tunes the response of the channel to nitric oxide and activation during *S*-nitrosylation is dependent on calmodulin at least for the RyR type 1 isoform in skeletal muscle (77). In addition, Favero and colleagues found that hypochlorous acid was more potent at activating RyRs in skeletal muscle than hydrogen peroxide (79). The way in which the RyR responds to channel inhibitors and activators is through a well-defined redox sensor intrinsic to the receptor protein (282). Calcium channel activators such as calcium and caffeine lower the redox potential of the protein that favors the oxidation of thiols and the opening of the channel while magnesium increases the redox potential, favors the reduction of disulfides, and the closure of the release protein. The redox sensor confers tight regulation of channel activity in response to changes in transmembrane redox potential produced by the levels of glutathione (87). The reader is referred to a number of excellent reviews on this topic (113, 182, 193, 203, 219)

IP_3 receptor function may also be regulated by reactive oxygen species but the conclusions are less clear than for the RyR. Oxidized glutathione and thimerosal increase the affinity of IP_3 for its receptor in retinal cells (165), while other reports state that thimerosal sensitizes the IP_3 receptor to IP_3 (32). The effects of oxidized glutathione on IP_3R's have been suggested to be indirect (213). Superoxide generated from hypoxanthine and xanthine oxidase stimulates IP_3-induced calcium release from SR in vascular smooth muscle by inhibiting degradation of IP_3 (244). Recent work has identified calcium entry through plasma membrane IP_3 receptors (58). These receptors may yet provide another target for regulation of calcium entry into cells under conditions of oxidative stress.

C. SERCA

Reversible *S*-glutathionylation of cysteine residues on SERCA pumps are believed to be involved in nitric oxide-dependent smooth muscle relaxation. Nitric oxide-derived peroxynitrite activates SERCA through oxidation of Cys674 (3). Other studies report inhibition of SERCA activity by hydrogen peroxide that can be attenuated with dithiothreitol (212), suggesting that hydrogen peroxide and nitric oxide may differentially regulate the activity of SERCA. Biological aging that is associated with a partial loss in SERCA activity, is also associated with a loss in oxidation sensitive cysteines on the SERCA protein (228).

D. PMCA

Oxidation of PMCA results in loss of activity. The loss of activity is proposed to occur as a result of altered coupling between the ATP binding domain and the autoinhibitory domain (195). This is considered to be an adaptive response to minimize ATP use during conditions of cellular stress. The inactivation of PMCA is also proposed to involve calmodulin. Following oxidation of methionine 144 and 145 on calmodulin, there is an enhanced binding affinity between the amino-terminal domain and the CaM-binding sequence of the PMCA that stabilizes PMCA in an inactive conformation (9, 49). Therefore, it has been proposed that calmodulin functions as sensor of oxidative stress. In addition, calmodulin is believed to protect PMCA from oxidation as binding of calmodulin to PMCA before exposure to hydrogen peroxide protected PMCA from oxidation suggesting that calmodulin can induce a conformational state resistant to oxidation (294).

E. Na^+/Ca^{2+} exchanger

Reactive oxygen species are associated with depressed myocardial contractility. This involves altered calcium handling that includes increased Na^+/Ca^{2+} exchange activity (99). However, the effects of reactive oxygen species on cardiac exchanger activity under conditions of oxidative stress may be indirect as mutant exchangers that do not contain the 15 endogenous cysteines behave like wild-type exchangers (190). An oxidative stress insult significantly decreases Na^+/Ca^{2+} exchange activity in synaposomal and microsomal brain preparations (152). However, for the brain K^+-dependent Na^+/Ca^{2+} exchanger, reduction of Cys395 located in the large intracellular loop stimulates the activity of the exchanger (42).

VIII. ROLE OF REDOX REGULATION OF CALCIUM CHANNELS IN DISEASE

Calcium is central to normal cellular function. It also plays a significant role in mediating pathology. In addition, ROS can interact with signaling pathways that possess regulatory function and have been implicated in mechanisms mediating disease. Therefore, understanding the effect of cellular redox state on calcium-dependent processes provides insight into mechanisms of disease that involve altered calcium homeostasis and altered ROS production. For example, an initial step in the development of atherosclerosis is the uptake of oxidized low density lipoprotein that then further stimulates the production of ROS and apoptosis (94). Under these circumstances, oxidative stress plays a part in the formation of nitric oxide-containing species that prevent vascular relaxation (and increase thrombosis). This process may be further exacerbated by calcium influx through L-type Ca^{2+} channels since dihydropyridine calcium channel blockers inhibit plasma and LDL oxidation in carotid and middle cerebral arteries of stroke-prone hypertensive rats (186). Calcium channel blockers also increased survival in the rats. Whether calcium influx through L-type calcium channels is a cause or consequence of increased ROS production and LDL oxidation under these circumstances is unclear but calcium participates in the pathophysiology. Oxidized LDL has been shown to increase calcium influx through the recombinant α_{1C} subunit of the L-type Ca^{2+} channel via enhanced production of ROS by the mitochondria (80). The enhanced production of ROS is proposed to occur as a result of effects of lysophosphatidylcholine on mitochondrial metabolism.

Hypoxic pulmonary vasoconstriction is an important mechanism by which pulmonary blood flow matches perfusion to ventilation and optimizes systemic oxygenation. Vasoconstriction occurs as a result of inhibition of K^+ channels by hypoxia that then results in a depolarization-mediated calcium influx through L-type Ca^{2+} channels, although a direct effect of hypoxia on a Ca^{2+}-dependent K^+ channel has been postulated (14, 205, 277).

Acute hypoxia (rapidly decreasing O_2 pressure to approx 15 mm Hg without depletion of cellular ATP) causes a decrease in basal L-type Ca^{2+} current in cardiac myocytes (Fig. 11) (118, 119, 120, 121, 232). This has also been demonstrated in systemic arterial myocytes and the recombinant α_{1C} subunit of the L-type Ca^{2+} channel, and the recombinant α_{1H} and α_{1I} subunits of the T-type Ca^{2+} channel (18, 82, 83, 85, 91). However, acute hypoxia potentiates calcium channels in a subset of resistance: pulmonary myocytes, the carotid body, and hippocampal neurons (18, 82, 83, 84, 90, 91, 119, 120, 163, 168, 169, 232, 242, 263). The reasons for the differential response by Ca^{2+} channels to acute hypoxia are unclear unless we examine the physiological significance. In carotid bodies, an influx of calcium through calcium channels is necessary for the release of neurotransmitter that signals the cardiorespiratory centre to increase respiratory rate and prevent systemic hypoxemia, while constriction of resistance pulmonary artery myocytes diverts blood to poorly oxygenated areas of the lungs to prevent ventilation perfusion mismatch (164). In cardiac myocytes, a decrease in calcium influx during the plateau phase of the action potential may be necessary to prevent arrhythmias associated with prolongation of the QT interval during hypoxia (46). In addition, a number of different splice variants are expressed in the heart. When identifying the hypoxia sensitive region, Fearon *et al.* found that only one variant was sensitive to hypoxia (Fig. 12) (85) Differential expression of hypoxia-sensitive splice variants may also explain why not all calcium channels display the same redox sensitivity. However, hypoxia also increases the sensitivity of the L-type Ca^{2+} channel to the β-adrenergic receptor agonist isoproterenol and this may not be beneficial to the cell (119, 120, 121). β-Adrenergic receptor stimulation is known to increase channel activity through activation of cAMP and subsequent protein kinase A-dependent phosphorylation of the α_{1C} subunit of the channel (175). This increases the mode 2 state of channel openings (long openings). An increase in the sensitivity of the channel to β-adrenergic receptor stimulation may explain the increased incidence of early afterdepolarizations associated with hypoxia and ischemia (119, 251). The increase in sensitivity of the channel to isoproterenol can be mimicked by dithiothreitol (119) and perfusing cells intracellularly with catalase (120). Consistent with this, exposing myocytes to the sulfhydryl oxidant phenylarsine oxide, is associated with a decrease in sensitivity of the channel to isoproterenol (229).

Even if the functional endpoint can be justified, the mechanisms by which acute hypoxia differentially regulate a calcium channel with the same molecular identity are unclear. It has been proposed that differential expression of mitochondrial electron chain components may explain the diversity in K^+ channel function and vessel response in pulmonary arteries versus renal arteries during acute hypoxia (178). In this case, the K^+ channels in pulmonary and renal arteries respond differentially to hypoxia due to the differences in expression of mitochondrial proteins in each cell type. This results in differences in mitochondrial production of reactive oxygen species and respiratory rate between renal and pulmonary artery myocytes. Renal artery and pulmonary artery myocytes will presumably be in different states of reduction/oxidation with differential production of reactive oxygen species

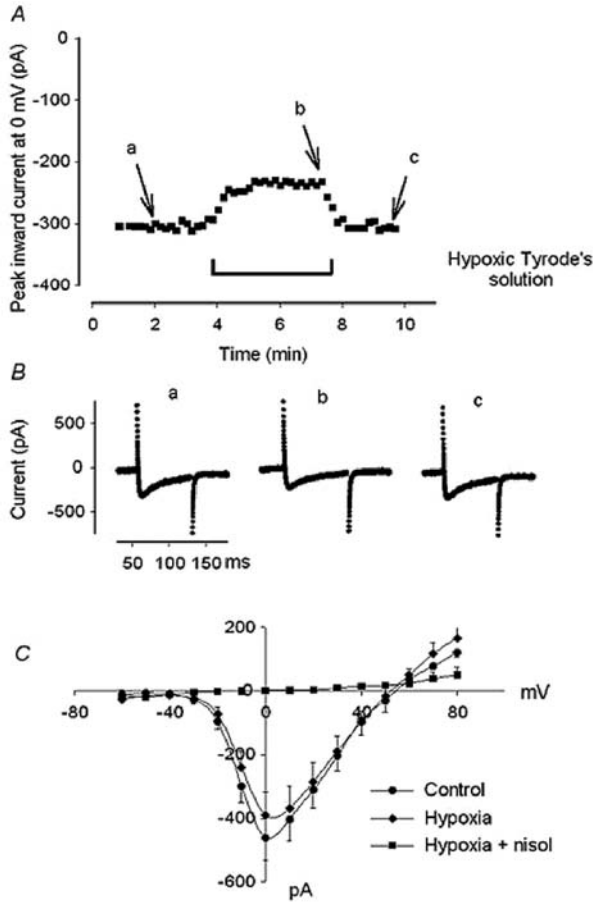

FIG. 11. Hypoxia inhibits the basal L-type Ca^{2+} channel in guinea pig ventricular myocytes. (**A**) Time course of changes in membrane current recorded during a 75 millisecond test pulse to 0 mV applied once every 10 second. Basal current recorded during exposure to hypoxic Tyrode's solution that is reversed upon switching the superfusate to Tyrode's solution containing room oxygen tension. (**B**) Membrane currents recorded at time points in protocol illustrated in **A**. (**C**) Mean ± SE current-voltage (I-V) relationship for 5 cells exposed to hypoxia and hypoxia + nisoldipine (nisol). Reproduced with permission from Ref. 119.

as the signal for the response. In the heart, it is apparent that ROS produced from the mitochondria regulate channel function. The effect of acute hypoxia on the L-type Ca^{2+} channel can be mimicked by dithiothrietol (119), perfusing cells intracellularly with catalase or the mitochondrial inhibitors FCCP or myxothiazol (120). In addition, pre-exposure of the myocytes to hydrogen peroxide attenuates the effect of catalase and the effect of hypoxia (Fig. 13). Therefore, hydrogen peroxide appears to play a central role in modulating channel function under conditions of hypoxia.

Since catalase mimics the effect of acute hypoxia, this would suggest acute hypoxia is associated with a decrease in cellular ROS production. A number of studies support this assertion (114, 120, 121, 149, 179, 277, 278). In adult ventricular myocytes a decrease in O_2 pressure from 150 mm Hg (room air) to 15 mm Hg results in a 41% decrease in superox-

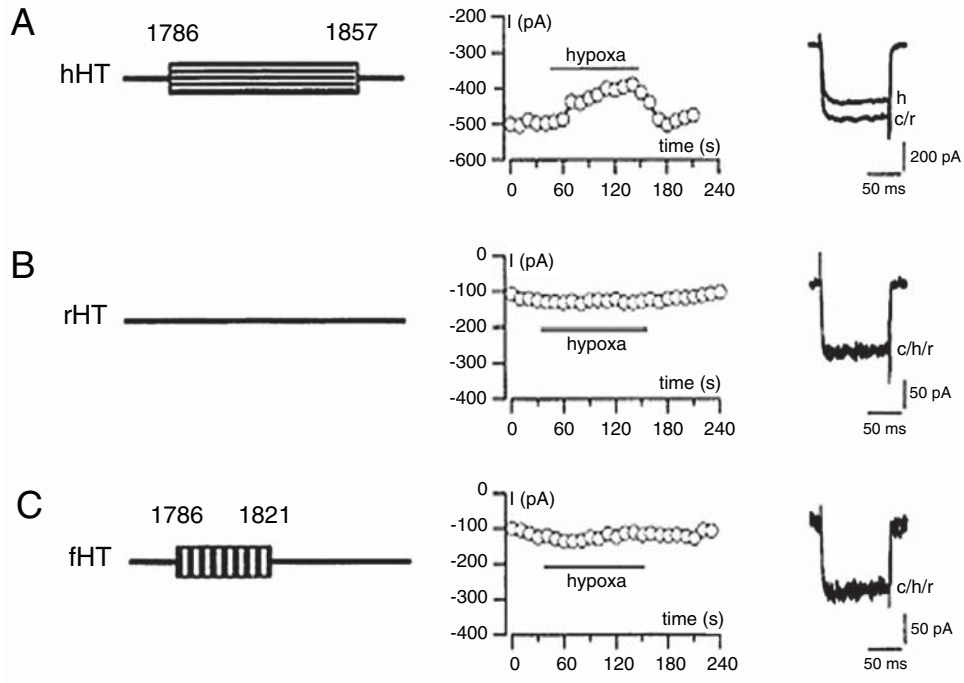

FIG. 12. Sensitivity of the hHT, rHT, and fHT isoforms of the recombinant human L-type Ca^{2+} channel α_{1C} subunit to hypoxia. Time course of changes in Ba^{2+} currents during exposure to 20 mm Hg hypoxia elicited in HEK 293 cells expressing hHT isoform (**A**), rHT isoform (**B**), and fHT isoform (**C**) of the α_{1C} subunit evoked by voltage steps to +10 mV from a holding potential of −80 mV. The portion of C-terminal region present in the isoform is shown on the *left*. Reproduced with permission. For further detail see Ref. 85.

ide (assessed with the fluorescent indicator dihydroethidium) (Fig. 14) (121) and a significant decrease in cellular hydrogen peroxide [assessed with 5- (and 6-) chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester] (120). The source of production of reactive oxygen species in cardiac myocytes, carotid body, resistance pulmonary arteries, and ductus arteriosis in response to acute hypoxia would appear to be the mitochondria and not NAD(P)H oxidase (15, 16, 119, 120, 121, 194).

However, whether hypoxia causes an increase or decrease in cellular ROS has been an area of apparent contradiction. It would appear that the conditions of hypoxia are an important determinant of ROS production. Prolonged periods of exposure of cardiac myocytes to hypoxia (>60 min) or hypoxia during contraction of myocytes (that is likely to cause severe hypoxia/anoxia or depletion of cellular ATP) appear to result in an increase in production of hydrogen peroxide and superoxide by the mitochondria (71, 95, 267). The reduced state of the electron transfer proteins, the mitochondrial membrane potential and the level and duration of hypoxia/anoxia are critical in determining production of reactive oxygen species in the mitochondria. Therefore, it is important to stress that not all hypoxic conditions are alike and cells appear to respond differentially to oxygen deprivation, depending on how ROS are generated in the cell, the cells' instrinsic requirements for oxygen, and the duration of hypoxia. Another reason for the apparent contradiction in reports regarding ROS production during hypoxia is the accurate detection of ROS. The high reactivity, variable diffusion rate across cell membranes, and the instability of ROS make them extremely difficult to detect in cellular systems. In addition, the methods of detection carry limitations. Typically, the assessment of ROS has been made using indirect measurements relying on the oxidation of probes or detector molecules to elicit fluorescent signals. Detection of superoxide has been made feasible using chemiluminescence substrates such as lucigenin and coelenterazine that can access intracellular sites of superoxide production and are relatively nontoxic to the cell. However, as with other methods of detection of superoxide such as paraquat and nitroblue tetrazolium (see Ref. 252 for review), lucigenin is capable of increasing superoxide in the xanthine oxidase/xanthine as well as the glucose oxidase/glucose reactions (157). Therefore, these detectors are nonspecific and rates of superoxide production may be underestimated. The two electron oxidation of dihydroethidium to the fluorophore ethidium bromide is relatively specific for superoxide but can also be oxidized by cytochrome *c*. Electron spin resonance has been used with the stable spin trap 5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide (DEPMPO) offering greater specificity for detection of superoxide. Hydrogen peroxide is typically assayed using horseradish peroxidase catalyzed assays or the fluorescence indicator 2′-7′-dichlorofluorescein (DCF). However, under certain conditions, these assays also lack specificity in that lipid peroxides and peroxynitrite can generate DCF fluorescence in the absence of hydrogen peroxide. Therefore, the use of more than one detection system may be necessary to yield the most reliable results.

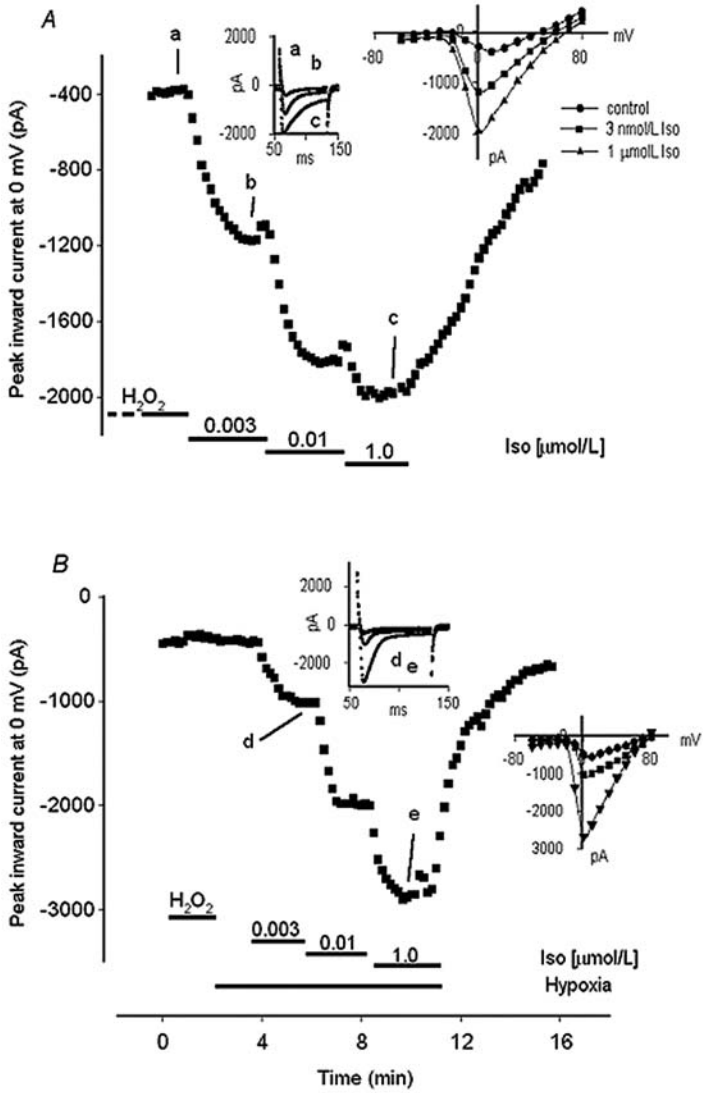

FIG. 13. Pre-exposure of guinea pig ventricular myocytes to H_2O_2 attenuates the effect of hypoxia and the effect of catalase on the sensitivity of the L-type Ca^{2+} channel to β-adrenergic receptor stimulation. During hypoxia (or when the cell is perfused intracellularly with catalase) the current produced in response to 3 n*M* isoproterenol would typically represent approximately 80% of the response elicited by a maximally stimulating concentration of the agonist (1 μ*M*) within the same cell. (**A**) Time course of changes in membrane current recorded in a cell during exposure to isoproterenol while being dialyzed with catalase. The cell was pre-exposed to 8.8 μ*M* H_2O_2 for at least 5 min prior to superfusion with isoproterenol in the absence of H_2O_2. Membrane currents recorded at the time points indicated are shown inset at *left*. I-V relationship in the same cell shown inset at *right*. (**B**) Time course of changes in membrane current recorded in a cell during exposure to hypoxia and isoproterenol. The cell was also pre-exposed to 8.8 μ*M* H_2O_2 for at least 5 min prior to superfusion with isoproterenol in the absence of H_2O_2. Membrane currents recorded at the time points indicated are shown inset at *left*. I-V relationship in the same cell shown inset at *right*. Adapted from Ref.

Internal calcium stores also respond to changes in oxygen tension. Calcium release from thapsigargin-sensitive internal stores or ryanodine receptors also contribute to neurosecretory and vasoconstrictor responses during acute hypoxia (6, 22, 67, 233, 271).

Channel responses to acute hypoxia are typically rapid and easily reversible. However, chronic hypoxia induces more permanent alterations in calcium channel function through altered channel gene expression. These changes have been implicated in aging and dementia (37, 81, 201, 275). These responses are not due to a direct effect of ROS on channel function, rather ROS are involved in increased formation of amyloid peptides that then increase the trafficking of calcium channels leading to altered calcium homeostasis. However, application of hydrogen peroxide to primary cultured neurons induces cell death as a result of calcium influx through transient receptor potential 2 (TRPM2) channels (137). It has been proposed that TRPM channels may play a significant role in mediating disease states such as diabetes and Alzheimer's dementia (89, 177). Hydrogen peroxide is also proposed to modulate synaptic plasticity via calcium channels (136, 148). The association of calcium channel subunits can be altered by the cell's redox state. TRPC3 channels associate with TRPC4 channels to form a redox-sensitive channel in response to the cell's redox state in endothelial cells (206).

Rapid reperfusion of the ischemic heart during coronary occlusion is vital to minimize the amount of myocardial damage. However, the reperfusion period imposes deleterious effects due to the generation of ROS. Contractile dysfunction or "myocardial stunning" has been linked to increases in cellular generation of ROS and includes impaired sarcoplasmic reticulum calcium handling and contractile responses (8, 31). Mice with targeted disruption of superoxide dismutase are vulnerable to ischemic damage (291), while overexpression of superoxide dismutase protects from postischemic injury (272). ROS produced during the reperfusion phase can trigger the release of further ROS from the mitochondria that is considered to be proarrhythmic in the heart (5, 13, 300). Mitochondrial ROS production is also dependent upon calcium uptake into the mitochondria such that an increase in calcium uptake is associ-

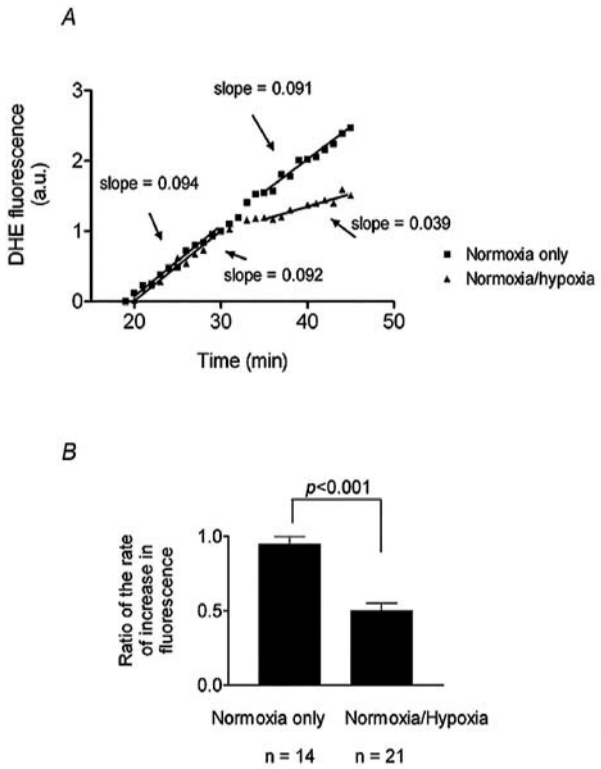

FIG. 14. Hypoxia is associated with a decrease in cellular superoxide. (**A**) DHE fluorescence recorded from a cell exposed to normoxia only and in another cell exposed to normoxia followed by hypoxia (normoxia/hypoxia). a.u.,arbitrary units. (**B**) Ratio of fluorescence for cells exposed to normoxia only and for cells exposed to normoxia followed by hypoxia (normoxia/hypoxia). Adapted from Ref. 121.

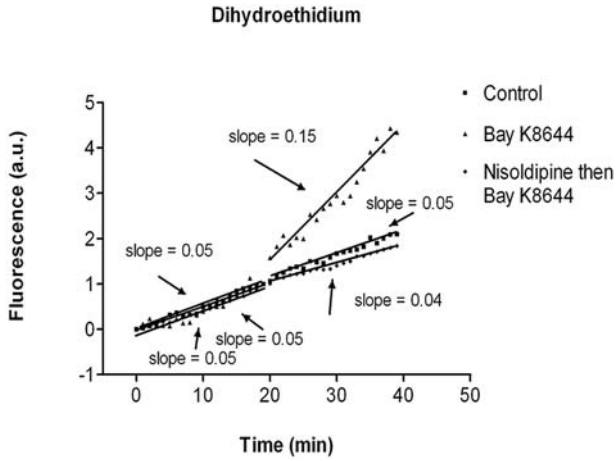

FIG. 15. Activation of the L-type Ca^{2+} channel is sufficient for an increase in cellular superoxide. DHE recorded from a cell prior to and following exposure to 2 μM Bay K8644 and in a cell prior to and following exposure to 2 μM nisoldipine, then 2 μM Bay K8644. Control, continuous recording in the absence of drugs in a cell.

ated with an increase in ROS release (36). We tested whether calcium influx through the L-type Ca^{2+} channel was sufficient for increased ROS production by the mitochondria in cardiac myocytes. We activated the channel with Bay K8644 and recorded changes in cellular superoxide (assessed with DHE) in the myocytes. Bay K8644 caused a 79% increase in DHE signal that was attenuated with 2 μ*M* nisoldipine (Fig. 15). The increase in DHE was also attenuated with 2 μ*M* Ru 360, an inhibitor of the mitochondrial calcium uniporter. This is good evidence that calcium entry through the L-type Ca^{2+} channel is sufficient for an increase in cellular ROS. Therefore, oxidation of thiol groups on the channel may cause sufficient calcium influx to result in a further increase in cellular ROS and induction of arrhythmia in the heart (5, 13, 300).

The development of cardiac hypertrophy is associated with activation of calcium-dependent hypertrophic signaling pathways such as ERK and MAP-kinase by ROS (135, 284). The source of ROS has been linked to chronic activation of NAD(P)H oxidase (39, 156). Hydrogen peroxide can regulate the activity of specific isoforms of protein kinase C in the heart that are implicated in the development of cardiac hypertrophy (34, 247, 248), and the transition from hypertrophy to failure (127). Abnormalities in calcium handling and contraction are well recognized in cardiac failure (99). Reverse mode Na^+/Ca^{2+} exchange due to increased hydroxyl radicals contributes to increases in intracellular calcium and diastolic dysfunction (296). The release of calcium due to oxidation of cysteines on ryanodine receptors has been implicated in mechanisms of cardiac preconditioning and activation of calcium-dependent gene expression (52, 117).

In vascular smooth muscle it is well recognized that ROS produced as a result of activation of NAD(P)H oxidase by angiotensin II are involved in the development of vascular smooth muscle hypertrophy and hypertension (264, 293). Antioxidants preserve cardiomyocyte morphology and contractile function in vascular disease associated with chronic diabetes (41). Calcium channel blockers decrease myocardial oxidative stress by upregulating Cu/Zn superoxide dismutase in stroke-prone hypertensive rats (261). In addition, superoxide is thought to mediate the effects of angiotensin II in the brain, including central regulation of blood pressure through activation of calcium channels (299).

IX. THERAPEUTIC OPPORTUNITIES

One approach to designing a therapeutic intervention to treat pathologies arising during oxidative stress is to target the reactive oxygen species. Where excessive production of ROS and oxidative stress induces pathology such as ischemia reperfusion injury in the brain and heart, treatment with antioxidants or overexpression of antioxidant enzymes have proven to be effective in animal models. As stated previously, mice with targeted disruption of superoxide dismutase are vulnerable to ischemic damage (291), while overexpression of superoxide dismutase protects from postischemic injury (272). Superoxide dismutase-deficient mice exhibit vascular dysfunction (59) while overexpression of catalase or superoxide dismutase retards atherosclerosis (288). Oral administration of vitamin E

(α-tocopherol) has been used in some trials and is attractive due to minimal side effects. However, clinical trials using antioxidant compounds have reported variable responses mostly with minimal effect (129). This may be due to an inability of antioxidants to effectively target reactive oxygen species isolated in organelles. For example, superoxide cannot cross plasma membranes and effects of the oxidant may be significant when produced in isolated organelles such as the mitochondria and Golgi or sarcoplasmic reticulum, which orally administered antioxidants cannot easily reach. The variable success of antioxidant trials may also be due to differences in risk in the patient cohort and variation in extent of disease.

Another approach to designing a therapeutic intervention is to target the source of generation of reactive oxygen species. Very effective and specific antagonists are being developed against NAD(P)H oxidase but they have only been used in experimental studies and their clinical efficacy has not been tested. One of these is the gp91ds-*tat* peptide inhibitor that prevents the formation of superoxide by uncoupling $gp91^{phox}$ from $gp47^{phox}$ (214). The inhibitor is effective in decreasing systolic hypertension associated with elevated angiotensin II in mice. Another drug readily available and used to treat raised uric acid levels is allopurinol. Allopurinol (and its active metabolite oxypurinol) prevents the production of superoxide by xanthine oxidase. Uric acid produced from the heart is considered a prognostic marker in patients with cardiac failure (218), suggesting that xanthine oxidase is active in heart failure. Consistent with this, xanthine oxidase inhibition has recently been shown to be effective in decreasing reactive oxygen species generation and calcium overload in ischemia-reperfused hearts (138) and reverse modelling in cardiomyopathic hearts (180, 240). A great deal of interest is now being directed towards developing novel xanthine oxidase inhibitors that may be useful in the treatment of cardiovascular and inflammatory disorders (103, 198). Interestingly, xanthine oxidase does not contribute to oxidative stress-associated endothelium-dependent vasodilatation with aging in humans (76). Finally, a predominant source of ROS in a number of cells is the mitochondria. However, the mitochondria would be difficult to target because much is still unknown about how and where the mitochondria produce reactive oxygen species both under normal and specific pathological conditions. In addition, complex activity, electron transport components, and mitochondrial membrane potential are intricately linked such that disrupting the activity of one component can have marked consequences on individual complex activity and oxygen consumption. Experimental interventions have been slow to develop. Therefore, a greater understanding of mitochondrial function under nonpathological conditions would help towards understanding abnormal function and designing interventional agents.

One of the challenges in designing therapeutic interventions that target channels directly is the varied responses of calcium channels to a stress. As described previously, calcium channels respond in a variety of ways to hypoxia. The response of calcium channels in one cell type may be adaptive but in other cells will be deleterious. For example, hypoxic pulmonary vasoconstriction prevents ventilation-perfusion mismatch in the lung by shunting blood to poorly perfused areas of the lung, but oxidative stress during ischemia reperfusion induces cardiac arrhythmia. Specific targeting of therapeutic agents to the cells inducing a deleterious response will be necessary to ensure that the therapy does not antagonize an adaptive response in another cell or organ. This challenge in drug delivery is being overcome with direct injection of drugs into the affected site or administration of drugs designed to allow delivery across the plasma or organelle membranes in cells. Unwanted side effects will occur if the drug is not specific in its site of action. Calcium channel antagonists are a good case in hand. They are used clinically in the treatment of hypertension, angina, and after myocardial infarction to decrease afterload. Side effects include postural hypotension, swelling in the ankles and feet, gingival hyperplasia, and constipation. In addition, calcium channel antagonists can cause cardiac arrhythmia and death by altering the duration of the action potential. The mode of action of the three classes of calcium channel antagonists, the phenalkylamines, dihydropyridines and benzothiazepines, is not clear. It is understood that the agents are use dependent and bind more effectively at depolarized potentials. Binding studies suggest they do not use the same receptor but enhance each other's binding (98). However, mutation studies suggest they all share overlapping determinants in the S6 transmembrane region of the α_1 subunit (see Fig. 1). Clinical studies have shown that a combination of calcium channel blockers and antioxidants may be effective in reducing atherosclerosis (187, 224) and in the prevention and treatment of Parkinson's and Alzheimer's disease (64, 274). Calcium channel blockers were more effective than angiotensin-converting enzyme inhibitors in prevention of stroke than protection from coronary heart disease in a large study of over 179,000 patients (269). Administration of verapamil before coronary perfusion with thrombolytic therapy improved recovery of the region around the infarct (204) and was associated with lower restenosis after percutaneous coronary intervention (24) In animals studies, calcium channel antagonists have been effective in reducing myocardial oxidative stress in stroke-prone hypertensive rats (261), protecting against myocardial reperfusion injury (273) and stunned myocardium (144). Therefore, the channel represents an attractive candidate for targeting. To treat disorders that involve acute modulation of channel function during alterations in redox state, a greater understanding of how the channel protein is affected is required. If a cysteine or cysteines are altered and identified, this would provide a potential site to target and modify channel function during pathological conditions. In addition, it is important to determine whether thiol group oxidation of the channel in response to oxidative stress is reversible or not. The vast majority of reported responses would suggest that they are. This implies that the intermediate formed is sulfenic acid as formation of sulfonic acid is irreversible. The reversible inhibition of tyrosine phosphatase 1B by hydrogen peroxide is a good example. This involves the formation of a sulfenic intermediate that is rapidly converted into a previously unknown sulfenyl-amide species, in which the sulfur atom of the catalytic cysteine is covalently linked to the main chain nitrogen of an adjacent residue (220). In addition, determining whether thiolation or glutathionylation of cysteines is involved would provide information on how cysteines are modified and assist with design of drugs. However, although cys-

teines are a likely target for redox regulation, they may not be the sole target as methionines or serines may also be sensitive to alterations in cellular redox state and confer altered channel function.

The physiological effects of an alteration in the cell's production of reactive oxygen species may be due to a direct alteration in the redox state of the channel protein or an indirect effect via alterations in the redox state of regulatory protein/s. More direct work identifying the cysteines on the channel protein that are altered as a result of a change in redox state would provide an opportunity to determine the role of the cysteine/s in channel function through site-directed mutagenesis. This would then help in designing drugs that could target specific cysteines allowing for specific modification of channel function appropriate to the pathological state.

X. CONCLUDING COMMENTS

Calcium channels play a fundamental role in regulating the electrical activity of cells and initiating intracellular responses. The physiology of many of these channels can be altered by the presence of ROS that can be produced from different sites within cells. The ROS most likely alter channel function by both altering the oxidation state of cysteines on the channel protein itself and by affecting regulatory intermediate proteins. Abnormal production of ROS arises in many disease states such as atherosclerosis, heart failure, ischemia-reperfusion, diabetes, hypertension, dementia, and aging. Thus, there is a significant desire to design therapeutic agents that either specifically target the ROS, the source of the ROS or the proteins (such as calcium channels) that are regulated by ROS. An understanding of the mechanisms by which ROS affect calcium channels and the sites on the protein that are involved in oxidation is only beginning to emerge. Greater knowledge of the structural details of calcium channel regulation by ROS is an important step in designing drugs that could target specific sites on the protein.

ACKNOWLEDGMENTS

Work presented in this review was supported by grants from the National Health and Medical Research Council of Australia. Dr Hool is the recipient of a National Health and Medical Research Council Career Development Award. Dr Corry is an Australian Research Council Postdoctoral Fellow.

ABBREVIATIONS

cADPR, cyclic adenine dinucleotide phosphate-ribose; cAMP, cyclic adenosine monophosphate; CRAC, calcium release activated current; ER, endoplasmic reticulum; GABA, γ-aminobutyric acid; GDP, guanosine diphosphate; HVA, high voltage activated channels; IP_3R, inositol triphosphate receptors; LVA, low voltage activated channels; MAP kinase, mitogen actived protein kinase; MCU, mitochondrial calcium uniporter; NFAT, nuclear factor of activated T cells; NMDA, *N*-methyl-D-aspartate; P-loop, pore forming loop of the alpha subunit of the calcium channel; ROS, reactive oxygen species; RyR, ryanodine receptors; SERCA, sarcoplasmic reticulum, endoplasmic reticulum calcium ATPase; SR, sarcoplasmic reticulum.

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Date of first submission to ARS Central, September 6, 2006; date of acceptance, November 20, 2006.