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Discoveries

COMPREHENSIVE INVITED REVIEW

Redox Control of Cardiac Excitability

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Abstract

Reactive oxygen species (ROS) have been associated with various human diseases, and considerable attention has been paid to investigate their physiological effects. Various ROS are synthesized in the mitochondria and accumulate in the cytoplasm if the cellular antioxidant defense mechanism fails. The critical balance of this ROS synthesis and antioxidant defense systems is termed the redox system of the cell. Various cardiovascular diseases have also been affected by redox to different degrees. ROS have been indicated as both detrimental and protective, *via* different cellular pathways, for cardiac myocyte functions, electrophysiology, and pharmacology. Mostly, the ROS functions depend on the type and amount of ROS synthesized. While the literature clearly indicates ROS effects on cardiac contractility, their effects on cardiac excitability are relatively under appreciated. Cardiac excitability depends on the functions of various cardiac sarcolemal or mitochondrial ion channels carrying various depolarizing or repolarizing currents that also maintain cellular ionic homeostasis. ROS alter the functions of these ion channels to various degrees to determine excitability by affecting the cellular resting potential and the morphology of the cardiac action potential. Thus, redox balance regulates cardiac excitability, and under pathological regulation, may alter action potential propagation to cause arrhythmia. Understanding how redox affects cellular excitability may lead to potential prophylaxis or treatment for various arrhythmias. This review will focus on the studies of redox and cardiac excitation. *Antioxid. Redox Signal.* 18, 432–468.

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I. Introduction

Partially reduced forms of oxygen (O_2) lead to the formation of oxygen free radicals, generally called reactive oxygen species (ROS). Partially reduced forms of nitrogen (N_2) are also described in living systems and are called reactive nitrogen species (RNS). The importance of ROS in physiological systems has been emphasized by several studies that determined that ROS and RNS could be deleterious or beneficial in living systems. Beneficial roles include cellular response against infectious agents, protection against reperfusion injury, and activation of a number of signaling pathways that regulate cellular process and responses (255). The deleterious effect of excessive ROS is called oxidative stress and can cause damage to the cellular lipids, proteins, and DNA, and thus inhibit normal functions (255). Several antioxidant systems exist to act as cellular mechanisms to protect against oxidative stress. A delicate balance of oxidants and antioxidants is necessary to maintain normal physiology in living cells.

Cardiac muscle tissue is characterized by automaticity and excitability. Cardiac excitability refers to the ease with which cardiac cells undergo a series of events characterized by sequential depolarization and repolarization, communication with adjacent cells, and propagation of electrical activity (22). The cardiac cell is coupled to this rhythmic excitability and contracts or relaxes in phase with the cardiac depolarization or repolarization. Excitability is the ability of a cardiac cell to generate an action potential at its membrane in response to depolarization and to transmit an impulse along the membrane. Cardiac contractility refers to the ability of a muscle tissue to contract when its thick (myosin) and thin (actin) filaments slide past each other in response to a stimulus. Since cardiac excitability and contractility are coupled, optimum and timely cardiac excitation is important for a proper contraction of the cardiac tissue, which may otherwise lead to various cardiac complications. Cardiac excitability arises from organized flow of ionic currents through ion-specific channels in the cell membrane, through the myoplasm and gap junctions that connect cells, and through the extracellular space (22). Each ion flow (current) possesses distinguishing kinetics, biochemical, or pharmacological properties, based upon the permeantion. These currents also determine the intracellular concentration of various ions and determine the potential across the cellular membrane (membrane potential).

The resting membrane potential of an adult cardiac myocyte is -90 mV, and with increased inward anionic currents, or decreased cationic currents, the membrane potential depolarizes. The upstroke of an action potential starts when the threshold potential of -55 to -60 mV has reached. The inward cationic and outward anionic currents maintain the plateau phase of the action potential, and gradually, the membrane potential decreases to negative voltages, and at the potential below -20 mV, voltage-dependent K^+ channels open to fully repolarize the cell. During the action potential, a normal cell completely loses its excitability (capacity to respond with new stimulus). The action potential period during which the cell looses its excitability is also called the effective refractory period (138). This refractory period in cardiac myocytes is $\sim$300 ms depending upon the rate of the heartbeat (138). The duration between two subsequent action potentials is called the relative refractory period. During this time, also the cellular excitability is lost. Thus, longer refractory periods would make the cells nonexcitable for a longer time. However, exceptions do exist where cells get excited during these refractory periods to generate a type of triggered arrhythmia called early afterdepolarizations (EADs) and delayed afterdepolarizations (DADs), which eventualy can cause arrhythmia. For years, it has been observed that the kinetic, biochemical, and pharmacological properties of the ion channels are affected by ROS/RNS (39, 91, 114, 146). Therefore, an appropriate balance of ROS/RNS and antioxidants is necessary for a proper cardiac excitability that may otherwise lead to a cardiac arrhythmia.

II. Overview of ROS Effects on Cardiac Ion Currents, Action Potential, and Excitability

In cardiac myocytes, rapid changes in ROS/RNS can contribute to excitability by means of altering the ion-handling properties of the ion channels, without affecting the fundamental contractile machinery of the myocytes. This was demonstrated in early studies in which 20–30 min of superfusion of guinea pig ventricular strips with a ROS-generating system caused $\sim$10-mV reduction in the resting membrane potential, $\sim$10-mV reduction in the potential amplitude, and $\sim$50 V/s fall in the rate of depolarization (211). Spontaneous activity in 50% of the ventricular strip preparations was abolished by adding an antioxidant (211). This has also been shown in some of the other studies where ROS, such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl), induced the transient augmentation of twitch amplitude in myocytes followed by unexcitability (129, 170). Similarly, various hydroperoxides depolarized the cardiac resting membrane potential, increased the generation of an action potential, and ultimately rendered the cells unexcitable due to modulation of various ion channels (199). However, these ROS-induced alterations of excitability were not accompanied by any effects on the myofilaments (199, 220), suggesting that the basic contractile machinery was not compromised. In ventricular cells from guinea pig hearts, ROS induced membrane depolarization with a concomitant increase in action potential generation. This was suggested to have occurred due to the inhibition of the inward rectifier K^+ channel activity and Ca^{2+} channel activity, by Nakaya *et al.* (199). Several studies confirmed that in general, exogenous ROS, such as H_2O_2, applied to isolated ventricular myocytes decreased the outward I_K, and/or increased the inward I_{Ca} and/or I_{Na} (23, 91, 123, 167, 211, 263). In 1993, Jabr and Cole (126) explained a ROS-induced loss of excitability in four stages. In stage one, an early 5–10-mV membrane depolarization increases the action potential duration due to decreased resting inward rectifying K^+ currents. In stage two, the activation of transient inward current mediated by a Na^+/Ca^{+2} exchanger (NCX) causes DADs and triggered activity (TA). In stage three, cardiac cells fail to repolarize, and thus continuously depolarize between -35 and -20 mV, due to increased cationic currents; and lastly, in stage four, shortening of the action potential duration and decreased hyperpolarization cause loss of excitability. The study also confirmed that these alterations in electrical activity could be prevented by a ROS scavenger, N-(2-mercaptopropionyl)glycine (126). The possible effects of ROS on ionic currents and excitation were further emphasized by application of the chemical oxidants

like tert-butyl hydroperoxide (t-BHP), dihydroxyfumarate (DHF), and xanthine/xanthine oxidase, which affected the K^+, Na^+, or Ca^{+2} currents and caused DADs and EADs in ventricular myocytes (3, 20, 200, 256, 262). Similarly, experimental inhibition of glutathione (GSH) synthesis with buthionine sulfoximine in canine hearts was associated with a decreased density of atrial I_{Ca} in myocytes (42), which would decrease the action potential duration. On the other hand, increasing the antioxidant capacity of the cells prevented the arrhythmogenic activity or proclivity of the cardiac tissue. For example, overexpression of catalase (CAT) prevented the oxidative stress-induced decrease in peak shortening, maximal velocity of shortening/relengthening, and increased time-to-90% relengthening in the mouse ventricular myocytes (64). A role for ROS toward cellular action potential generation, excitability, and associated cardiac diseases has also been observed clinically. Specifically, the levels of GSH are now said to be associated with the cardiovascular events in humans. For example, the risk of cardiovascular events is inversely associated with increasing quartiles of glutathione peroxidase (GPx) 1 activity in patients (32), and in myocytes from the atrial fibrillation patients, the GSH levels are reduced relative to controls (42). Moreover, atrial myocytes isolated from patients with atrial fibrillation when superfused with N-acetylcistein (NAC) (a clinically used antioxidant) showed increased L-type calcium currents (42). It can be inferred that the atrial fibrillation occurred due to decreased I_{Ca} that might shorten the action potential and increase the excitability.

Overall, as ROS affect the ion-handling properties of the Ca^{+2}, Na^+, and K^+ channels, it is expected that it will also affect the action potential of the cardiac cells by altering I_{Ca}, I_{Na}, or I_K. Indeed, ROS-induced membrane depolarization occurred with increased Na^+ window current (the window current results from the overlap of activation and inactivation steady-state curves due to depolarized membrane voltages that are sufficient to transiently open a few available channels) (27), increased activity of NCX (90), inhibition of the inward I_K (199), activation of an inwardly directed nonselective cationic current (182), increased intracellular Ca^{+2} concentration associated with increased activity of the L-type Ca^{+2} channels (117) or ryanodine receptors (RyRs) (9), and decreased activity of the sarcoplasmic reticulum (SR) Ca^{2+} pump SERCA2a (149). An example from guinea pig myocytes (Fig. 1A–C) shows ROS-mediated increased I_{Ca} and I_{Na} and decreased I_K (44, 167, 249). These alterations in currents cause prolongation of the action potential and EADs (44). In contrast to this example, shortening of the action potential duration has also been observed in an ischemic heart and is attributed to ROS-mediated increase in the delayed rectifying I_K, activation of ATP-sensitive K^+ (K_{ATP}) channels (mitochondrial or sarcolemal), or other mitochondrial K^+ channels (30, 199, 275). In addition, ROS induced decreased cytosolic Ca^{+2} associated with decreased activity of L-type Ca^{2+} channels (100) and RyRs (287), or increased activity of NCX (219) has also been shown in other studies, and this may cause shortening of the action potential followed by loss of excitability as shown by Jabr and Cole (126). The contradictory results on ROS effects on ion channels and the action potential might be due to species differences (44, 276) in the concentration (89) and the type of ROS involved (41) and the number and type of channels affected (12, 149). In support of this idea, Tokube

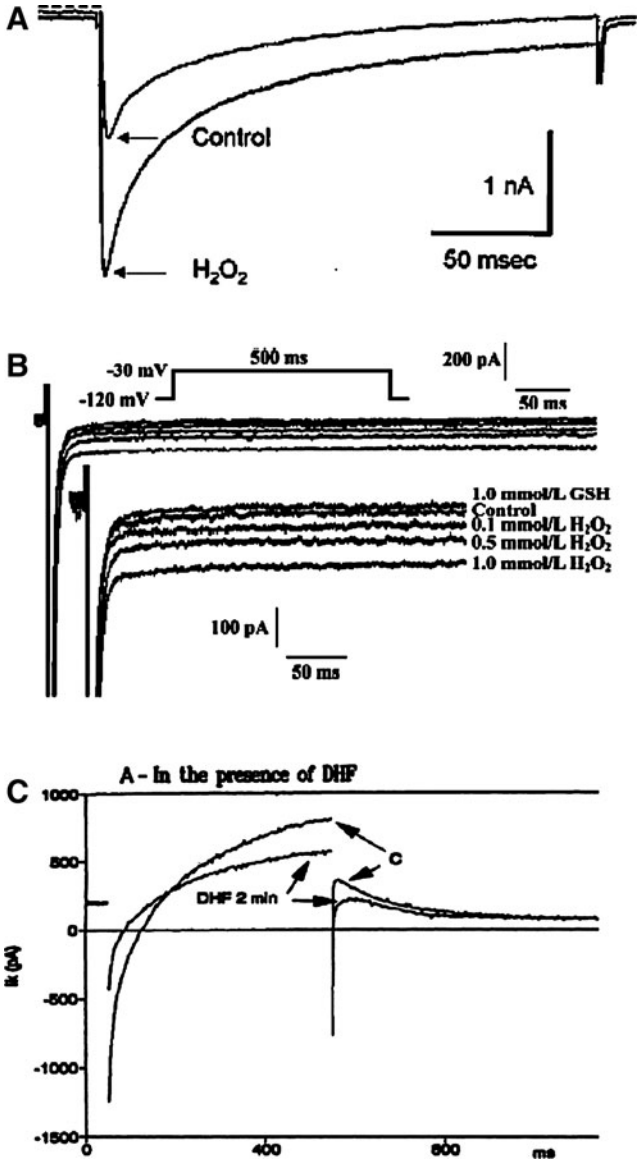

FIG. 1. Examples of the effects of reactive oxygen species (ROS) on various channel currents. (A, B) Hydrogen peroxide (H_2O_2) increased the Ca^{+2} currents from the L-type Ca^{2+} channels (I_{CaL}) and the persistent Na^+ currents from the cardiac Na^+ channels (I_{NaP}). **(C)** Dihydroxyfumarate (DHF), an oxidant, decreased the inward rectifier K^+ currents (I_{Kr}) from the Kv11.1 channels. All these currents were measured in the guinea pig myocytes, and are taken from (44, 167, 249), with copywrite permissions, respectively. The *arrows* indicate differences in the currents.

et al. (250) reported ROS-induced biphasic changes in the action potential duration, with initial lengthening of the action potential due to a rapid decrease in whole-cell I_K and subsequent shortening due to a decrease of whole-cell I_{Ca} and increase in ATP-sensitive outward K^+ current (I_{KATP}). Apart from these time-dependent ROS-mediated increased or decreased Ca^{+2} concentrations, ROS-induced oscillation in intracellular Ca^{+2} concentrations has also been implicated in arrhythmogenic afterdepolarization (180) that would eventually lead to loss of excitability. These oscillations might be related to the ROS modulation of the RyR that are primarily

responsible for the Ca^{+2}-induced Ca^{+2} release in the cells. More recently, ROS-induced opening of the mitochondrial channels has been implicated in causing shortening of the action potential and thus arrhythmia (40). In this regard, Zhou *et al.* modeled a whole cell to demonstrate that increasing ROS production from the mitochondria triggers limit-cycle oscillations of the mitochondrial membrane potential ($\Delta\Psi_m$) and mitochondrial respiration, causing transient periods of mitochondrial uncoupling. These transient periods of mitochondrial uncoupling decrease the cytosolic ATP/ADP ratio and activate sarcolemal I_{KATP} that shortens the cellular action potential duration and ultimately suppresses excitability (296).

Given this overview, it can be seen that ROS effects on various ion channels and the interplay among these channels may affect cardiac action potential generation and thus excitability in complex ways. Therefore, pathophysiological processes that increase ROS synthesis might cause arrhythmogenic electrical dysfunctions and nonexcitability of the cardiac tissue. This review will first outline various ROS, their synthesis, their metabolism by antioxidants, and their roles in various pathways in the cardiomyocytes, followed by a more comprehensive review of the effects of ROS on various ion channels, individually, and finally, additional considerations for cardiac electrophysiology and arrhythmia.

III. ROS in Cardiac Physiology

A. Types of ROS and their synthesis in the heart

The major ROS in cardiac cell are superoxide radical ion ($O_2^{\bullet -}$), singlet oxygen ($O_2^{\bullet}$), H_2O_2, hydroxyl radical ($OH^{\bullet}$), and HOCl (176). The major sources of oxidative stress in cardiovascular system involve the enzymes (i) xanthine oxidoreductase (XOR), (ii) NADPH oxidase, (iii) nitric oxide synthase (NOS), and (iv) the mitochondrial cytochromes; and (v) hemoglobin (176). Various ROS in a cell and their synthesis are shown in Figure 2. The addition of one electron to dioxygen forms the superoxide anion radical ($O_2^{\bullet -}$). Since mitochondria consume most of the O_2 in the cells for their respiratory chain, the production of superoxide occurs mostly within the mitochondria of a cell.

The transfer of electrons within the constituents of the mitochondrial respiratory chain is shown in Figure 3. In this respiratory chain, a small percentage of electrons prematurely leak to O_2 at Complexes I, II, or III, resulting in the formation of the toxic $O_2^{\bullet -}$ (Fig. 3). For a direct evidence of ROS

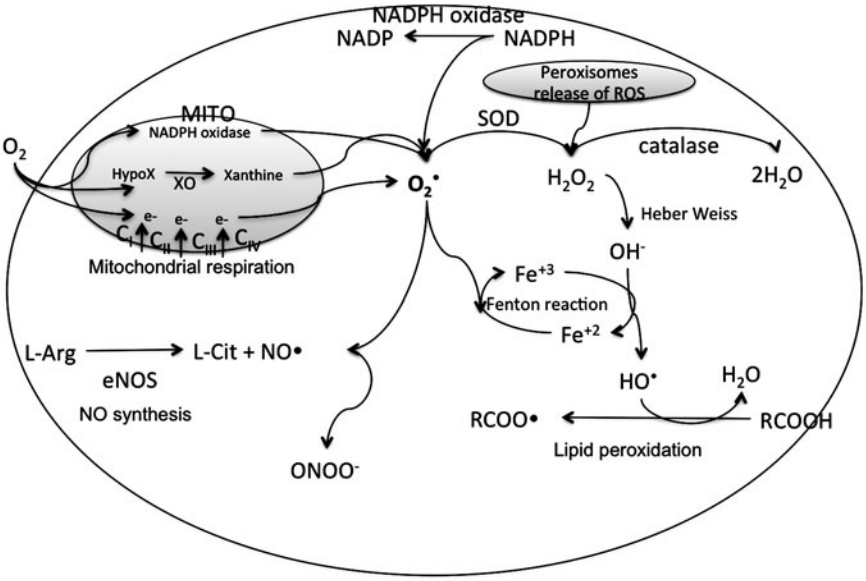

FIG. 2. Major ROS synthesis pathways in cardiac myocytes. In the mitochondria (MITO), a small percentage of electrons from the respiratory chain prematurely leak to O_2 at complexes I, II, or III, resulting in the formation of the toxic superoxide radical ion ($O_2^{\bullet -}$). $O_2^{\bullet -}$ is also generated by release of a free electron from the reactions where NADPH is oxidized to NADP by NADPH oxidase, and hypoxanthine is oxidized to xanthine (X) by xanthine oxidase (XO). The $O_2^{\bullet -}$ generated in the mitochondria can freely pass through the mitochondrial membrane into the cytosol. Cytosolic $O_2^{\bullet -}$ can interact with other intracellular molecules to generate (hydroxyl radical [$OH^{\bullet}$], a neutral form of the OH) from H_2O_2. This H_2O_2, but not $O_2^{\bullet -}$, is mainly generated in the peroxisomes of the cells. When peroxisomes are damaged, their H_2O_2-consuming enzymes are downregulated, and H_2O_2 releases into the cytosol (62). In a Haber–Wiess reaction, $O_2^{\bullet -}$ reacts with H_2O_2 to form $OH^{\bullet}$ and a hydroxyl anion (OH^-). Moreover, under conditions of metabolic stress, iron-containing molecules in a cell, such as 4Fe-4S cluster-containing enzymes, release free iron. This iron participates in a Fenton reaction generating $OH^{\bullet}$ radical. The Haber–Wiess and the Fenton reactions occur in conjunction, because the OH^- released from the Haber–Wiess reaction feeds into the Fenton reaction to form more $OH^{\bullet}$. The reduction of Fe by $O_2^{\bullet -}$ yielding Fe^{2+} and O_2 further propels the Fenton reaction. Other radicals derived from O_2 that can be formed in living systems are lipid peroxyl radicals ($RCOO^{\bullet}$) that are synthesized due to peroxidation of the membrane lipids (6). Overproduction of nitrogen species is called nitrosative stress. Reactive nitrogen species (RNS) are formed from a single nitrogen radical ($NO^{\bullet}$) generated in cells by nitric oxide synthase (NOS), which metabolizes L-arginine (L-Arg) to L-citruline (L-Cit) with the formation of $NO^{\bullet}$ (145). $NO^{\bullet}$ and $O_2^{\bullet -}$ react together to produce a much more potent oxidative molecule peroxynitrite anion ($ONOO^-$). The figure was created by adapting information from (176, 195, 255), respectively.

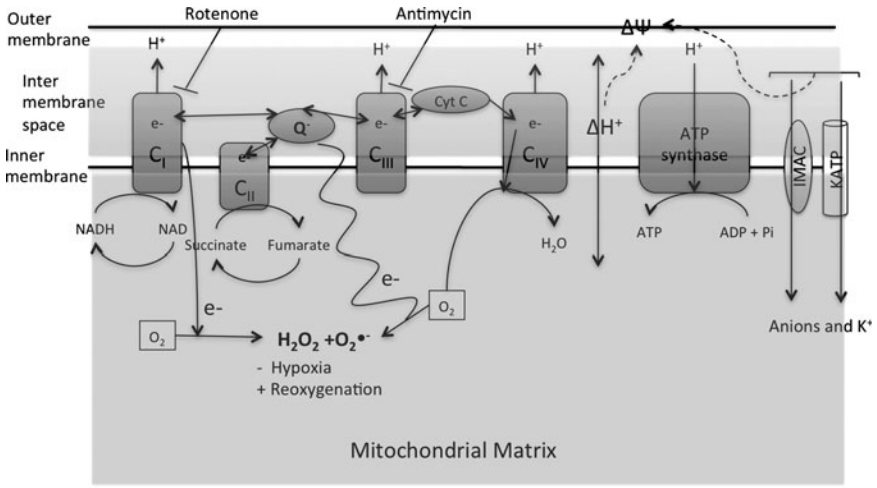

FIG. 3. Synthesis of superoxide radical in the mitochondria. Electron transfer occurs between the Complex I through Complex IV of the respiratory chain. At the mitochondrial inner membrane, electrons from NADH (at Complex I) and succinate (at Complex II) pass to the coenzyme Q (ubiquinone [UQ]). UQ passes electrons to Complex III (cytochrome bc1 complex), which then passes the electrons to the cytochrome c (cyt c). Cyt c passes electrons to Complex IV (cyt c oxidase), which uses the electrons and hydrogen ions to reduce molecular oxygen to water. This enzymatic series produces a proton gradient across the mitochondrial membrane, producing a thermodynamic state that has the potential to do work. However, in this respiratory chain, a small percentage of electrons prematurely leak to O_2 at complexes I, II, or III, resulting in the formation of the toxic $O_2^{\bullet -}$. Reverse electron flow from Complex I also generated superoxide radicals. Inhibitors of Complex I and III that enhance the generation of ROS in cardiac mitochondria are Rotenone and Antimycin, respectively. Mitochondrial membrane potential ($\Delta\Psi_m$) is determined by the proton gradient across the membrane. Mitochondrial ion channels such as inner membrane anionic channel (IMAC) and ATP-sensitive K^+ (K_{ATP}) channels cause ionic flux through the membrane and contribute toward mitochondrial membrane potential. The figure was created by adapting the information from (106, 195, 215), respectively.

generation from the cardiac mitochondria, the synthesis of H_2O_2 from the isolated mitochondria from hearts, or mitochondria in intact cardiac cells, can be determined in a fluorescence-based assay (106). Within these fluorescence-based assays, inhibition of the mitochondrial respiratory chain at any stage causes generation of large amounts of ROS (Fig. 3). For example, in isolated heart mitochondria from beef or rodents, inhibition of Complex III by antimycin (218), or Complex I by rotenone (241), produces large amounts of ROS. Inhibition of the Complex III causes stabilization of the ubisemiquinone radical (218), which transfers a single electron to O_2 to form $O_2^{\bullet -}$ in the intermembrane space in the mitochondria, whereas inhibition of Complex I produces $O_2^{\bullet -}$ from NADH (108). When uninhibited, the maximum rate of $O_2^{\bullet -}$ production by Complex III is generally smaller than that by Complex I. Moreover, it has also been confirmed that the primary site of $O_2^{\bullet -}$ generation in the mitochondrial electron transport chain is flavin mononucleotide of Complex I *via* a reverse electron flow, not forward electron flow *via* ubiquinone of Complex III (164, 241). Thus, it is generally accepted that *in vivo* Complex I is the major source of superoxide. In addition to respiratory complexes, many other enzymes associated with cardiac mitochondria have been shown to produce $O_2^{\bullet -}$ or H_2O_2 under various conditions. For example, mitochondrial ROS production is increased by high levels of the xanthine reductase (60) or NADPH oxidase (192).

Regardless of the source of $O_2^{\bullet -}$, the mechanism and quantity of $O_2^{\bullet -}$ generated are dependent on the experimental substrate and energetic conditions (106, 183). For example, an elevated O_2 concentration will also promote superoxide synthesis, as the rates of the nonenzymatic reactions of O_2 with radical intermediates are proportional to the local O_2 concentration (35). This is because larger proton gradient across the inner membrane causes increase in the rate of respiration, and thus increases utilization of the O_2 (183). Hypoxia, as in ischemia, causes a decreased ROS production because of the decreased availability of O_2. A decreased O_2 availability halts the respiratory chain, decreases the proton gradient that further decreases the O_2 utilization, and thus depolarizes the mitochondrial membrane potential (12, 183). The less utilization of O_2 may form trace amounts of ROS. However, reoxygenation/reperfusion causes sudden burst of O_2 into the mitochondria with a partially inactive respiratory activity, leads to an access to O_2 unutilized by the respiratory chain, and thus causes a huge increase in the ROS production (144). This increased and decreased ROS synthesis with hypoxia/reoxygenation is associated with the modulation of the activities of several ion channels, alterations in action potentials, excitability, and thus arrhythmia (12, 183). In addition, such conditions favor $O_2^{\bullet -}$ production from Complex I by enhancing the reverse electron transport (106), and may also act by increasing the lifetime of the ubisemiquinone radical at Complex III (236). In this regard, mitochondrial ion channels, in particular ATP-sensitive K^+ channels (K_{ATP} channels), may serve an important role in ROS generation in cardiac myocytes, and consequences of this increased ROS on myocyte excitability have been recently described (39). In summary, ROS are primarily synthesized in the mitochondria, depending upon the availability of the O_2, and several components of the respiratory chain and ion channels are involved in this process. These ROS increase the overall cardiac oxidative stress leading to damage. This oxidative damage results whenever the ROS produced by mitochondria

evade detoxification. This detoxification occurs due to the presence of the antioxidant systems as discussed below.

B. Antioxidants protect myocytes against oxidative stress

Defense mechanism against free radical-induced oxidative stress involves enzymatic and nonenzymatic antioxidant defense systems. Enzymatic antioxidant defenses include superoxide dismutase (SOD), GPx, and CAT. Nonenzymatic antioxidants are ascorbic acid (vitamin C), alpha-tocopherol (vitamin E), GSH, carotenoids, flavonoids, and other antioxidants (255). In addition, dietary antioxidants such as polyunsaturated fatty acids (PUFAs) and their effects on cardiac contractility and excitability have been extensively studied. Various antioxidants and how they regulate oxidative stress are shown in Figure 4. Under normal conditions, there is a balance between both the intracellular antioxidants and oxidants levels maintained by the activities of the antioxidant enzymes. For example, GPx reduces the cellular peroxides in a reaction in which GSH acts as a cofactor and gets oxidized oxidized GSH (GSSG) (Fig. 4) (179). GSSG is accumulated inside the cells, and the ratio of GSH/GSSG is a good measure of oxidative stress of an organism. GSH is highly abundant in the cytosol (3–15 m*M*) and mitochondria (5–11 m*M*), and is the major soluble antioxidant in the cell compartments (179). The levels of GSH in cultured rat heart cells were found to be about 148 nmoles/mg of protein, and these levels fall with stress (85). Levels of GSH are associated with the cardiovascular events in humans as well (32). Besides regulating other oxidants in the cell, GSH/GSSG also directly reduces the disulfide bond formed within cysteine residues of the proteins by serving as an electron donor, and has been shown to alter all the major ion channel activities in a cardiac cell (146). GSH is found almost exclusively in its reduced form, since the enzyme, GSH reductase, which reverts it from its oxidized form, is constitutively active as well as inducible upon oxidative stress. Because GSH is synthesized in the cytosol, its mitochondrial presence requires an active transport across the mitochondrial membrane. However, recently, it has been shown that externally added GSH is readily taken up by the mitochondria despite the 8 m*M* GSH present in the mitochondrial matrix (214). One of the possible mechanisms of this process is that the increased GSH levels can maintain the mitochondrial K_{ATP} channels in a closed state, thereby maintaining the mitochondrial respiration, and thus decreased synthesis of ROS (214).

The second major antioxidant is SOD. In humans, there are three forms of SOD: (i) a homodimeric copper–zinc SOD (CuZnSOD) found primarily in the cytosol, (ii) a homotetrameric glycosylated CuZnSOD in the extracellular space, and (iii) a homotetrameric manganese SOD (MnSOD) in the mitochondrial matrix (184). Several lines of evidence suggest that the mitochondrial (MnSOD) plays a major role in limiting the synthesis of the ROS in the mitochondria of the cardiac cells (283) (see Fig. 4). For example, transgenic mouse lines expressing increased levels of human MnSOD in the cardiac mitochondria had decreased ROS levels and mitochondrial damage compared with the nontransgenic littermates, when treated with Adriamycin that increases mitochondrial ROS generation (283). Thus, increased activity or expression of SOD will prevent ROS-induced damage to the cardiac functions. However, clinical studies indicate that there is no relationship between the total SOD activity in erythrocytes and the risk of cardiovascular events (32). The impact of CAT, another major antioxidant, on cardiac dysfunction and oxidative damage was studied in transgenic mice that overexpressed CAT in a cardiac-specific manner (64). Increasing the blood glucose levels increased ROS generation in cardiac mycocytes of wild-type mice, but not the CAT-overexpressing mice (64). The increased ROS in control mice were associated with the intracellular Ca^{+2} changes and a decreased Ca^{+2} clearance, which was abrogated by the overexpression of CAT (64). Therefore, these results indicate that perhaps, ROS affected the activity or expression of various Ca^{+2} channels in the cardiac cells, in particular the sarcoendoplasmic reticulum Ca^{+2} ATPase (SERCA) channels and NCX that are responsible for removing Ca^{+2} from the cytosol (reviewed in the later sections), and that CAT overexpression abrogated this ROS effect. Increased intracellular Ca^{2+} concentrations do affect the action potential and excitability without affecting the contractile machinery (172) (also discussed later). In this regard, it is important to consider that CAT itself does not alter the activity or expression of the NCX, SERCA, or phospholamban (a protein that regulates the activity of the SERCA) (64). Such studies have shown that the GPx, SOD, and CAT are important in maintaining the oxidative stress of the cardiac myocytes, and thus regulate the cardiac membrane potential, action potential, and excitability indirectly by limiting the ROS-induced alteration of ionic balance. Other important antioxidant systems that are important in various tissues are thioreductase and methionine sufoxide reductase. Recently, two novel human thioredoxin reductase isoenzymes were cloned and sequenced, one mitochondrial and the other one

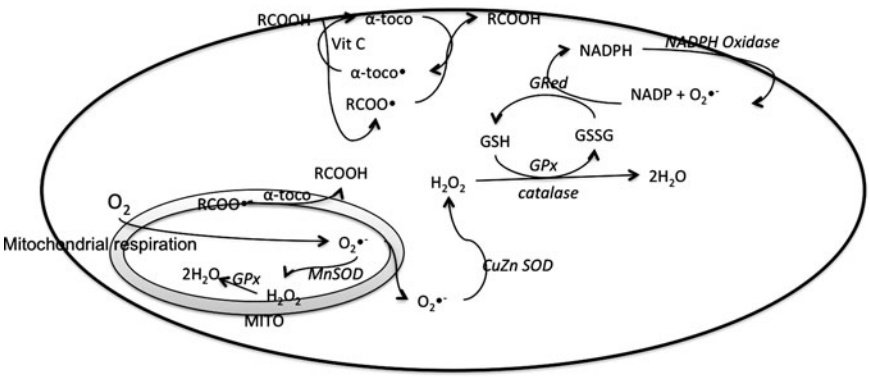

FIG. 4. Antioxidants in the cardiac cells. The major antioxidants are glutathione (GSH)/oxidized glutathione (GSSG) along with the enzymes glutathione reductase (GRed) and glutathione peroxidase (GPx), catalase, superoxide dismutase (SOD), NADPH oxidase, and α-tocopherol and vitamin C. MITO, mitochondria; $RCOO^{\bullet}$ and RCOOH, lipid radical and lipid; α-$toco^{\bullet}$ and α-toco, α-tocopheroxyl radical and α-tocopherol.

predominantly expressed in testis. The major substrate for thioredoxin reductase is thioredoxin, which is as an electron carrier necessary for the catalytic cycles of biosynthetic enzymes and also protects cytosolic proteins from aggregation or inactivation *via* oxidative formation of intra- or intermolecular disulfides. Since many ion channels possess oxidizable cysteine residues, appropriate levels of reduced thioredoxin in cardiomyocyte are important, and to maintain this level, an active thioredoxin reductase is necessary. Paradoxically, thioredoxin reductase may possibly be (transiently or permanently) inactivated by oxidants such as H_2O_2. In cardiac myocytes, the thioredoxin/thioreductase system has an important implication on regulation of ion channel activity against ROS (discussed later under each ion channel section). For example, thioredoxin influence on various K^+ channel activities has been studied (159, 160). Methionine sulfoxide reductase (MsrA) reverses oxidation of methionine in proteins. As shown by the use of an MsrA knockout (KO) mice, lack of MsrA in cardiac myocytes reduces the myocyte capability against ROS stress due to a significant increase in cellular oxidized protein levels resulting in a dysfunction (201). MsrA KO mice also revealed that under oxidative stress or physical stress, cellular contractility is significantly decreased (201). Conversely, overexpression of MsrA in primary neonatal rat cardiac myocytes protected myocytes against hypoxia-/reoxygenation-induced oxidative damage (216). Another study showed that the calmodulin (CaM) kinase II (CaMKII) can be activated by ROS directly without the Ca^{2+}-CaM complex if MsrA is absent or inhibited (73). This activated CaMKII has many detrimental implications on various ion channels and ion fluxes in the myocytes (will be discussed in the later sections). Therefore, MsrA is related to myocardial apoptosis, impaired cardiac function, and increased mortality after myocardial infarction.

Among the nonenzymatic antioxidants, α-tocopherol has been studied. Because of its hydrophobic nature, almost all the cellular α-tocopherol is present in the membrane fraction of cells. In that location, it can readily donate an electron to a fatty acid hydroperoxyl radical, thus breaking the chain reaction associated with lipid peroxidation (132). Evidence suggests that the resulting α-tocopheroxyl radical can be recycled back to α-tocopherol by cytoplasmic ascorbic acid, and by ubiquinol in microsomes and mitochondrial membranes (132). It has been shown that increased lipid peroxides in the isolated myocytes caused an increase in the repolarizing I_K, and thus in the arrhythmic phenotype, and that treatment with α-tocopherol prevents the I_K modulation by reducing the lipid peroxide content of the cells (130). Dietary antioxidants, specially PUFAs, may act as antiarrhythmic agents principally, because they reduce Ca^{2+} entry by blocking I_{Ca}, act directly to decrease I_{Na}, and act indirectly to reduce the intracellular Ca^{2+} transients (267). For further details of the mechanism of action of PUFA and other dietary antioxidants, readers are encouraged to refer the literature.

IV. Redox Balance Affects Cardiac Physiology

The delicate balance of the reductants and the oxidants in a cell is called redox balance or simply redox. The ROS-induced oxidative stress in cardiac myocytes has been linked to various diseases such as ischemic heart disease, cardiomyopathies, cardiac hypertrophy, congestive heart failure, and arrhythmia (147). On the other hand, protective concentrations of ROS have been shown to be important in decreasing the postischemia/reperfusion damage to the heart (95). These paradoxical roles of ROS are explained by the findings that ROS can affect multiple pathways (protective or harmful), either by affecting the activities of various proteins/secondary messengers or by acting as a signaling molecule itself. For example, extracellular signal-regulated kinase (ERK), mitogen-activated protein kinase (MAPK), and specific isoforms of protein kinase C (PKC) are regulated by ROS in the cardiac cells (52, 61, 95, 194), and these may participate in favorable or detrimental pathways. These signaling molecules may be involved in the pathways that are responsible for either a short-term effect by modulating the activities of other proteins such as ion channels (289) or long-term effects by modulating the activities of transcription factors such as cJun that would ultimately alter the protein expression (52). Various signaling molecules and associated pathways affected by ROS in the cardiac cells are shown in Figure 5. Alterations of these signaling pathways are involved in pathogenic mechanisms for arrhythmia, hypertrophy, heart failure, and postischemic injury (61, 95, 194). In as far as direct effects are concerned, ROS alter the structural conformation of the proteins (including ion channels) by oxidizing thiols or nitrosylation of cysteines to modulate their functions. The effect of ROS on various ion channels is tabulated in Table 1.

It is important to recognize that all ion channels are made up of macromolecular complexes involving many proteins. For example, the human cardiac Na^+ channel, $Na_V1.5$, which is a member of the family of voltage-gated sodium channels (Na_V1 to 9), consists of a primary α-subunit and a secondary β-subunit (18). In a mammalian system, the α-subunit of $Na_V1.5$ is sufficient to generate sodium currents; however, the β1-subunit increases the density of currents and regulates the gating of the channels (18). In addition, phosphorylation/dephosphorylation of α- or β-subunits, by the action of PKC, PKA, or Ca-CaM kinase, is important for activity of the Na^+ channels (82). More importantly, ROS affect both the α- and the β-subunits either directly or indirectly to eventually alter I_{Na} and thus cellular excitability (163, 167, 263). Similarly, cardiac Ca^{2+} channels (L-type or the T-type) are formed by the combination of as many as 5 subunits, α_1, α_2, β, γ, and δ. The β subunit increases channel expression and accelerates the activation and inactivation kinetics. The α_1-subunit is exclusively expressed in cardiac tissue, and its phosphorylation promotes a slow gating mode of the Ca^{2+} channels, increasing Ca^{2+}entry, and resulting in cytotoxicity (74). In addition, a loss of function of the α_1- or β_{2b}-subunit of the L-type Ca^{2+} channels has been associated with many cardiac diseases. Needless to say, ROS-mediated direct or indirect modulation of the structure or functions of either of the subunits will alter the cardiac I_{Ca}, action potential, and thus excitability. The third major type of the ion channels, the K^+ channels, is a complex group of channels divided into three major categories: the voltage-gated K^+ channels, inward rectifier K^+ channels, and the background K^+ currents (155). Just like the Na^+ and the Ca^{2+} channels, most of the K^+ channels also consist of α-subunits and single/multiple β-subunits. The channel functional units also include the complementary proteins K^+ channel-associated protein (KChAP) and the K^+ channel-interacting protein (KChIP), which may increase channel activity and alter channel kinetics (94). K^+ channel functions are regulated by various cellular proteins through processes such as phosphorylation and dephosphorylation (178). Once again, ROS affect the functions of various K^+ channels by

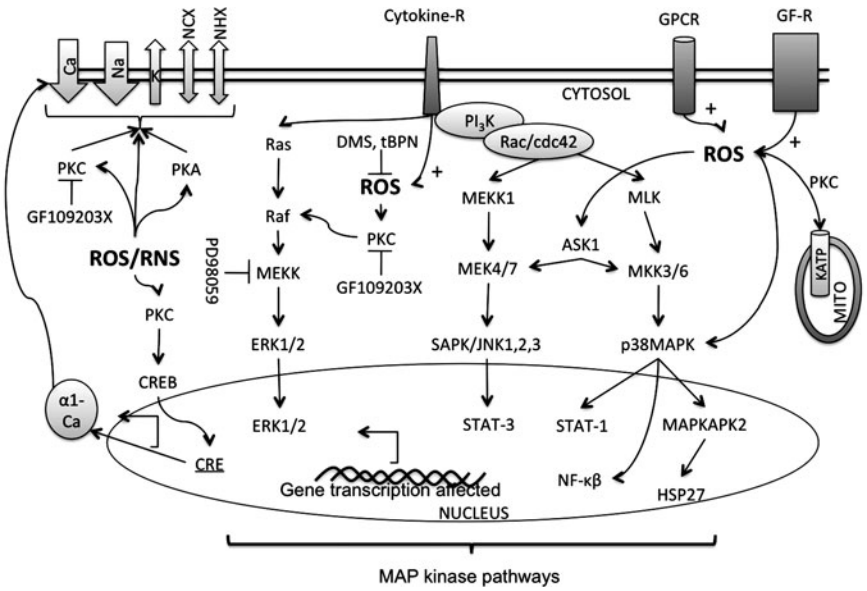

FIG. 5. Pathways affected by ROS in cardiac myocytes. Apart from the hypoxia/reoxygenation-mediated ROS-synthesis, ROS are also produced after activation of cell surface receptors such as the cytokine receptors (Cytokine-R), the G-protein-coupled receptors (GPCR), and the growth factor receptor (GF-R) such as TGF-β1 (135, 290). Cellular ROS, such as H_2O_2, activate the three subfamilies of mitogen-activated protein kinases (MAPKs), namely the stress-activated protein kinases/c-Jun N-terminal kinases (SAPKs/JNKs), the extracellularly responsive kinases (ERK1/2), and p38-MAPK (52). ROS activate ERK in a protein kinase C (PKC)-dependent manner because the activation of ERKs can be inhibited by PD98059 [which inhibits the activation of ERK kinases (MEKKs)], and also by the PKC inhibitor GF109203X. ROS also activates p38-MAPK that then activates the downstream MAPK-activated protein kinase 2 (MAPKAPK2) that in turn phosphorylates HSP25/27 (52). Free-radical trapping agents such as dimethyl sulfoxide (DMS) and N-t-butyl-a-phenyl nitrone (tBPN) inhibit the activation of MAPKAPK2 (52). ROS were estimated as lipid peroxides, activate apoptosis-regulating signal kinase-1 (ASK-1), and Rac/cdc42 pathways. Activation of ASK-1 and Rac/cdc42 leads to activation of other kinases such as MEKK, SAPK/JNK, p38MAPKs, and Jun kinases, and this is reversed by addition of the reducing agents (290). Activation of such kinases activates several downstream nuclear transcription factors as NFkβ, STATs, and HSP. Therefore, dominant negative mutant ASK-1 can attenuate the agonist-mediated activation of NFkβ in cardiac cells in response to increased ROS (225). Activation of such transcription factors as Jun and NFkβ will lead to an altered gene expression. Indeed, DNA microarray studies show induction of nearly 100 genes in response to oxidant stress (203). Various ion channels such as Ca^{2+}, Na^+, and K^+ channels as well as Na^+/Ca^{+2} exchanger (NCX) and Na-H exchanger (NHX), and ATP sensitive K^+ channels in the MITO are affected by these pathways. Inhibitors of MEKK, PKC, and ROS are shown.

directly or indirectly modifying the subunit functions or structures, therefore reducing the repolarizing I_K (84, 146, 160, 202, 244, 256). ROS may alter the structure and function of the channel-forming proteins by direct interaction (S-nitrosylation or formation of disulfide bonds) (50). Such direct modulations are normally irreversible and harmful to the cells.

The protective role of ROS in cardiac cells [as in ischemic hearts (95)] is exemplified by the ROS-induced activation of guanylyl cyclase (GC), which causes formation of the well-characterized second messenger cyclic guanosine monophosphate (cGMP) (187). The apparent paradox in the roles of the protective ROS as an essential biomolecules in the regulation of cellular functions and as toxic ROS causing damage, at least in part, is related to differences in the concentrations of ROS produced. This is analogous to the effects of NO, which has both regulatory functions and cytotoxic effects depending on the enzymatic source and relative amount of NO generated. NO functions as a signaling molecule mediating vasodilation when produced in low concentrations by the eNOS in vascular endothelial cells (212) and as a source of highly toxic oxidants utilized for microbial killing when produced in high concentrations by inducible NOS in macrophages (171). Thus, while discussing ROS effects, it is imperative to consider the mode of ROS activation (direct or indirect), and the source, concentration, and duration of ROS activity.

As mentioned earlier, ROS modulate a variety of signaling pathways in cardiac myocytes as shown in Figure 5. This interaction with the cell-signaling pathway occurs through a direct interaction with the thiol group of the regulatory proteins that may be essential secondary messengers and are necessary for a variety of normal cellular functions. Most important of these proteins are the kinases, phosphatases, and other transcription factors. Recent articles on the effects of ROS on various kinases/phosphatase signaling can be found in the literature (197). The most studied and important of these proteins is PKC. ROS-activated PKC in the cardiac cells regulates Ca^{+2} handling by activating L-type Ca^{+2} channels (mainly by affecting open probability) (151), phosphorylates, and thus activates Na^+ channels (264), sarcolemmal Na^+–hydrogen exchanger (16), NCX (232), and K^+ channels (196). PKC-, and not PKA-, mediated activation of the L-type Ca^{+2} channels in adult ventricular myocytes depends on the activation of the ERK1/2 pathway (235). Similarly, the human inward rectifier K^+ channel currents (I_{K1}) in atrial cardiomyocytes and one of its underlying ion channels, the $Kir_{2.1b}$ channel, are inhibited by PKC-dependent signal transduction pathways, possibly

Table 1. Effect of Reactive Oxygen Species on Various Cardiac Ion-Channel Currents

Currents	*ROS system applied*	*Experimental systems*	*Effects on channel currents*	*References*
I_{CaL}	H_2O_2 application	Isolated guinea pig ventricular myocytes or recombinant Cav1.2 in HEK293 cells	Increased	(117, 257)
	Hypoxia (decreased mitochondrial ROS)	Isolated guinea pig ventricular myocytes or recombinant Cav1.2 in HEK293 cells	Decreased	(76, 113, 117)
	HOCl application	Isolated hamster ventricular cardiomyocytes	Decrease	(100)
	Oxidizing agent, thimerosal, or buthionine sulfoxime	Isolated guinea pig or canine ventricular myocytes	Decrease	(42, 150)
RyR	H_2O_2 application or application of oxidizing agents such as DTDP or DTNB	Reconstituted channels formed by the cardiac RyR purified from pig heart, or in intact rat cardiac myocytes, or in sheep myocardium,	Increased the open probability and increase in Ca^{2+} spark activity	(9, 63, 67, 278, 286)
	GSNO and CysNO	Cardiomyocytes	Increased channel activity	(242)
SERCA	H_2O_2 application	microsomes from HEK-293 cells overexpressing SERCA2b, or rat cardiomyocytes	Decreased activity	(96, 149)
	Chemical NO application	SERCA reconstituted in phospholipid vesicles	Increased activity	(2)
	Unaffected I_{Kto} or I_{K1} by direct application of oxidants as t-BHP or H_2O_2 (27, 263)			
I_{K1}	ROS generated from the xanthine/xanthine oxidase system or H_2O_2	Guinea pig cardiac ventricular myocytes	Decreased	(53, 199)
	Diamide	Rat ventricular myocytes	Un affected	(159)
Kv channels; Shaker K^+ channels (Kv1.3, Kv1.4, and Kv1.5), one Shaw channel (Kv3.4)	Photoactivation of rose bengal	Expressed in *Xenopus* oocytes	Decreased	(66)
(KShIIIC, KShIIID and HukII	H_2O_2 application	Expressed in *Xenopus* oocytes	Inhibition of the time-dependent fast inactivation	(256)
I_{Ks} (Kv7.1)	H_2O_2 perfusion or diamide application	Expressed in CHO cells or in rat cardiac myocytes	Unaffected	(84, 159)
		Expressed in Calu-3 cells	Increased	(58)
I_{Kss} (steady state outward K^+ currents	DHF		Decreased	(44)
I_{peak} (Kv4.2, Kv4.3, or Kv1.4) and I_{Kss} (Kv1.5 and Kv2.1)	Diamide application	Ventricular myocytes of rats	Decreased	(160)
I_{Kto}	BCNU and BSO treatment of rats	Ventricular myocytes of rats	Decreased	(159)

(*continued*)

Table 1. Continued

Currents	*ROS system applied*	*Experimental systems*	*Effects on channel currents*	*References*
I_{Kr} (Kv11.1)	$FeSO_4$ and ascorbic acid generated ROS	Expressed in *Xenopus oocytes*	Increased outward currents Unaffected inward currents	(244)
	H_2O_2 application	Expressed in the CHO cells	Increased outward currents Decreased inward currents	(23)
	Hypoxia induced ROS generation	Kv11.1 α-subunit expressed in HEK	Decreased conductance	(202)
I_{KCa} (BK_{Ca})	H_2S application	BK_{Ca} in rat glomus cells and the human recombinant BK_{Ca} channel expressed in HEK293	Reduced conductance	(248)
I_{KATP}	Hypoxia H_2O_2 or GSSG application	Isolated myocytes	Increased	(123, 250, 277)
I_{Na} (I_{peak} or I_{NaT})	NADH application, hypoxia or application of GSSG	HEK cells stably expressing human cardiac Na^+ channel subunits	Decreased	(163, 262)
	Exogenous NO application	HEK cells stably expressing human cardiac Na^+ channel subunits	Increased	(3, 253)
I_{NaP}	H_2O_2, hypoxia or application of GSSG		Increase late	(167, 237, 238, 263)
IP3R	H_2O_2, GSSG, t-BHP application	In endothelial cells or in permeabilized hepatocytes	Increased activity	(71, 72, 107, 294)
NCX	H_2O_2 application, or hypoxia reoxygenation	In rabbit ventricular myocytes	Increased activity	(70, 90, 149)
Na^+/K^+ ATPase	Xanthine plus xanthine oxidase, or hypoxia. [Cardiac glycosides have also been shown to increase cellular ROS (111, 271)]	In rat and bovine myocytes	Increased activity	(271, 272)

I_{CaL}, L-type Ca^{2+} currents; RyR, ryanodine receptors; I_{NaP}, persistent or late sodium currents; I_{NaT}, transient or peak sodium currents; I_{Ks}, slow component of delayed rectifier K-current; I_{K1}, inward rectifier K-currents; I_{Kto}, transient outward K-currents; I_{Kss}, steady-state outward K-currents; NCX, Na^+/Ca^{2+}-exchanger; IP3R, IP3 receptor; SERCA, sarcoendoplasmic reticulum Ca^{+2}-ATPase; I_{KATP}, K_{ATP} channel current; I_{KCa}, calcium-activated K-currents; BCNU, 1,3-bis-(2-chloroethyl)-1-nitrosourea; BSO, buthionine sulfoximine; CysNO, S-nitrocysteine; DHF, dihydroxyfumarate; DTDP, 2,2′-dihydropyridine; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); GSNO, S-nitrosoglutathione; GSSG, oxidized glutathione; HOCl, hypochloric acid; H_2O_2, hydrogen peroxide; ROS, reactive oxygen species; t-BHP, tertbutyl hydroperoxide.

contributing to arrhythmogenesis in patients with a structural heart disease in which PKC is activated (133). An isoform of PKC, PKCε, also activates the mitochondrial K_{ATP} channel (188). Another kinase, PKA, has been shown to activate the cardiac Na^+ channels in the similar fashion; however, it phosphorylates a different site on the channels and then the PKC phosphorylation site (295). Furthermore, PKC activates the MAPK pathway that will ultimately lead to the increased transcription of the proteins. In fact, ROS increased the transcription of the human α1-subunit of cardiac Ca^{+2} channels in a PKC-dependent manner (251).

V. Redox Effect on Cardiac Calcium Channels and Excitability

The intracellular Ca^{+2} concentration is regulated by several types of channels, pores, and exchangers that work in a synchronous manner (114) (Fig. 6). It is well known that the concentration of intracellular calcium $[Ca^{2+}]_I$ regulates cardiac contractility *via* its interaction with the contractile machinery that includes troponin, tropomyocin, myosin, and actin (114). However, the role of intracellular calcium toward cardiac excitability is relatively underappreciated. $[Ca^{2+}]_I$ affects cellular excitability through direct or indirect modulation of I_{Ca}, I_K, or I_{Na} *via* proteins as CaM or CaMKII that in turn are affected by ROS. While L-type Ca^{2+} channels are responsible for influx of Ca^{2+} into the cells, their activation and facilitation also depend on the initial $[Ca^{2+}]_I$ (102). Initial increase in the local $[Ca^{2+}]_I$ causes the formation of the Ca^{2+}-CaM complex, which then activates autophosphorylation of the CaMKII (120). The activated CaMKII phosphorylates L-type Ca^{2+} channels and RyRs to increase $[Ca^{2+}]_I$ (120, 233). Since ROS increase Ca^{2+} currents by directly modulating the α- and/or β- subunits of L-type Ca^{2+} channels to increase local $[Ca^{2+}]_I$, and since this initial increase in $[Ca^{2+}]_I$ activates CaMKII that then activates more L-type Ca^{2+} channels, it could be expected that ROS facilitate I_{CaL} *via* CaMKII activation. Indeed, H_2O_2 increased I_{CaL} amplitude and slowed inactivation of I_{CaL}, and this facilitation of I_{CaL} was abolished by a CaMKII blocker KN-93 (239). Further investigations revealed that CaMKII-dependent phosphorylation of α1C subunits of L-type Ca^{2+} channels at S1512 and S1570 mediates this I_{CaL} current facilitation (31). Thus, ROS-mediated activation of CaMKII would increase $[Ca^{2+}]_I$, which could depolarize the cells and affect the cellular excitation. In addition, ROS-mediated CaMKII activation due to initial Ca^{2+} increase also increases persistent Na^+ currents (I_{NaP}) to prolong APD, and thus reduces the time that a cell can be excited again. For example, in rabbit myocytes, both acute and chronic CaMKIIδ (the predominant form in the heart) overexpression shifted voltage dependence of Na^+ channel availability by -6 mV and slowed their recovery from inactivation (259) in a Ca^{2+}-dependent manner. CaMKIIδ also increased I_{NaP}, and thus the total intracellular Na^+ concentration $[Na]_I$. This effect was completely prevented by CaMKII inhibition in the case of acute CaMKIIδ overexpression (259). Wagner *et al.* also reported that CaMKIIδ-mediated changes occurred due to a direct interaction of CaMKIIδ with Na^+ channels and a subsequent phosphorylation of Na^+ channels (259). Thus, $[Ca^{2+}]_I$ levels regulate the I_{Na} *via* CaMKII. Indeed, *in vivo*, transgenic CaMKIIδ overexpression prolonged the QRS duration and QT intervals due to an increase in the I_{NaP}, which would prolong the effective refractory periods, and thus decrease the time that a cell can be excited again (259). In contrast to these results, in guinea pig ventricular myocytes, CaMKII (in the pipette solution) shifted the voltage dependence of Na^+ channel availability by approximately +5 mV and enhanced the recovery from inactivation. CaMKII also increased persistent Na^+ currents, thereby increasing the intracellular Na^+ levels. Moreover, the Ca^{2+}-CaM complex (in the pipette solution), in absence of CaMKII, also increased the voltage dependence of Na^+ channel availability and the fraction of channels undergoing slow inactivation, but it did not alter the magnitude of the late current (5). Investigations have confirmed that CaM binds to the carboxy-terminal IQ domain[1] of the human cardiac Na^+ channel in a Ca^{2+}-dependent manner (246).

Although the two studies mentioned above reported a number of contradictory results, they reported one consistent result. In both of the studies, CaMKII increased the persistent Na^+ currents that would increase the $[Na^+]_I$. These experiments indicate that a local increase in $[Ca^{2+}]_I$ prolongs the effective refractory period by increasing either I_{CaL} or I_{Na}. The prolongation of the effective refractory period will reduce the availability of the cell to be excited again to generate a full action potential. Under abnormal circumstances, any depolarization during this prolonged effective refractory period will generate EAD that may be passed to the adjacent tissue that is not in its refractory period. This may cause arrhythmia. Possible explanations for controversial results in the two studies mentioned above might be the use of a different CaMKII isoform, species differences, or a possible existence of more than one Ca^{2+}-dependent mechanism regulating the activity of Na^+ channels. Nevertheless, these two studies did show that $[Ca^{2+}]_I$ affects the Na^+ channel activity, but did not emphasize the direct role of ROS. In this regard, Xie *et al.* reported that H_2O_2 treatment of rabbit ventricular myocytes increased $[Ca^{+2}]_I$ (268), which enhanced the peak and late I_{CaL} and increased SR Ca^{+2} release. This acute sarcoplasmic Ca^{2+} release may cause an increased CAMKII activity that will then lead to an increased I_{NaP} (259). Thus, ROS-induced increase in I_{CaL} and I_{Na} will prolong the action potential duration (prolongs the effective refractory period) to decrease the excitability. Intracellular Ca^{+2} levels and its interaction with CaM also affect voltage-gated K^+ channels. K^+ currents through the KCNQ1 (Kv7.1) channels are inhibited by KCNE4 (a β-subunit), which interacts with CaM in a Ca^{2+}-dependent manner (51). Disruption of the KCNE4-CaM interaction either by mutagenesis or by acute Ca^{2+} chelation impairs the ability of KCNE4 to inhibit KCNQ1, and thus increased the I_K (51). Thus, in presence of ROS-induced increased $[Ca^{2+}]_I$, it can be assumed that the Ca^{2+}-CaM complex will inhibit the Kv7.1 currents to prolong the relative refractory period and give rise to DADs. However, this needs to be tested directly. Nevertheless, it is clear that cellular Ca^{2+} levels regulate the refractory periods (by altering the activity of various channels) and alter cellular excitability. Thus, ROS effects on various Ca^{+2} channels are reviewed below.

A. Effect of ROS on the voltage-dependent L-type Ca^{+2} channel

Cardiac L-type Ca^{+2} channels are composed of polypeptide subunits (α1, β, and α2/δ) and form a heterotetrameric complex with a molecular mass of about 400 kDa (43). The

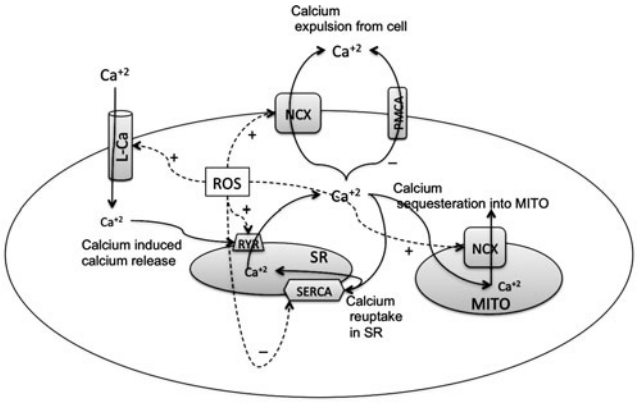

FIG. 6. Effect of ROS on various Ca^{+2} handling channels in cardiac myocytes. Depolarization causes opening of the voltage-gated L-type Ca^{2+} channel (L-Ca) on sarcolemmal membranes, which produces the entry of a small amount of Ca^{+2} that triggers the opening of another Ca^{+2} channel on the sarcoplasmic reticulum (SR) called the ryanodine receptors (RyRs). This process, known as calcium-induced calcium release (CICR), increases the total Ca^{+2} content in the cytoplasm. Intracellular Ca^{+2} is decreased by reuptake into the SR by the sarcoendoplasmic reticulum Ca^{+2} ATPase (SERCA) pump and removed from the cell by NCX (114, 298). Each of these Ca^{+2} regulators is affected by the ROS in the cells to increase or decrease the cellular Ca^{+2}. ROS induce the Ca^{+2} currents from the L-Ca, the Ca^{+2} release from RyR, and expulsion of Ca^{+2} out of the cytosol through the NCX on the sarcolemmal and mitochondrial membranes. ROS decrease the Ca^{+2} sequestering by SERCA or expulsion of Ca^{+2} by the plasma membrane Ca^{+2} ATPase (PMCA) pump.

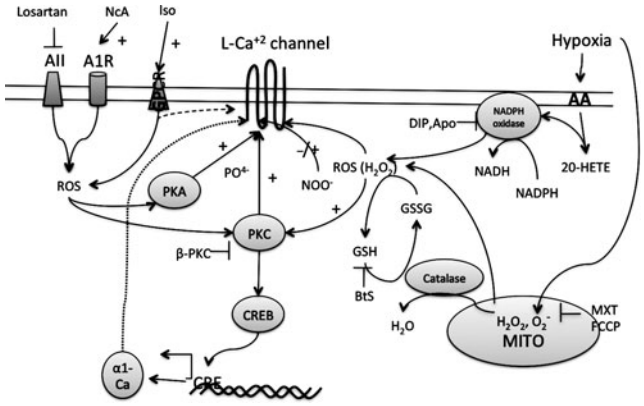

FIG. 7. ROS induces the activity of L-type Ca^{+2} channels. Activation of various receptors such as angiotensin (AII) receptor, adenosine receptor (A1R), or GPCR increases ROS in the myocytes. Cytosolic ROS activate PKC or PKA that activates the channels by phosphorylation. PKC also activates CRE-binding protein (CREB) that binds to cAMP-response elements (CRE) on DNA to induce the expression of α-1 subunit of the L-type Ca^{+2} channels. BtS, buthionine sulfoxime; MXT, myxothiazol; FCCP, carbonylcyanide p-trifluoromethoxyphenylhydrazone, a protonophore and uncoupler of mitochondrial respiratory chain; DIP, diphenylene iodonium; Apo, apocynin; 20-HETE, 20-hydroxyeicosatetraenoic acid; AA, arachidonic acid; NcA, N-acetylcysteine; Iso, isoproterenol.

hydrophobic α1 polypeptide is entrenched in the cell membrane, whereas the β-subunits are located in the cytoplasm. The δ-subunit is anchored in the cell membrane and has a single transmembrane segment with a short intracellular part and a long glycosylated extracellular part. The α2 peptide is an extracellular subunit of the Ca^{+2} channel (43). The L-type Ca^{+2} channels are involved in the ROS-induced modulation of Ca^{+2} currents by several pathways that may precipitate arrhythmia (Fig. 7). The Ca^{+2} channel activity was reduced when the pore-forming α1C-subunit of the human L-type Ca^{+2} channel, expressed in the HEK 293 cells, was exposed to acute hypoxia (76). Similarly, acute hypoxia decreased basal L-type Ca^{+2} channel currents (I_{CaL}) in isolated guinea pig ventricular myocytes without shifting the current–voltage relationship (76, 113). Hypoxic effects may be mediated by a decreased generation of ROS in the mitochondria, because ROS generation is directly proportional to the local concentration of O_2 available in the mitochondria (195). In guinea pig ventricular myocytes, direct H_2O_2 application or mitochondrial ROS (confirmed with inhibitors of mitochondrial respiration) increases the basal I_{CaL} and sensitivity of I_{CaL} to isoproterenol (257). This provided direct evidence of the role for the mitochondria and ROS generated by the mitochondria in regulation of the I_{CaL} and suggests that an increase in ROS will increase the I_{CaL}. This has been further confirmed by direct application of ROS (such as H_2O_2) to measure I_{CaL}. H_2O_2 perfusion of the HEK 293 cells expressing the α1C-subunit of the human L-type Ca^{+2} channel (117) or the guinea pig ventricular myocytes (257) showed significantly enhanced depolarization-evoked Ca^{+2} currents in a voltage-dependent manner. Application of CAT to these cells/myocytes reduced the I_{CaL} currents (117, 257). It was also revealed that the hypoxia effects on I_{Ca} were not due to the generation of mitochondrial H_2O_2 (117). Similarly, oxidized LDL has been shown to increase Ca^{+2} influx through the recombinant α1C-subunit of the L-type Ca^{2+} channel *via* enhanced production of ROS by the mitochondria (75). Contrary to this, application HOCl to isolated hamster ventricular cardiomyocytes caused a dose-dependent decrease in peak I_{CaL} without affecting apparent reversal potential, activation, and inactivation kinetics (100). In addition, the NADPH oxidase inhibitors diphenylene iodonium (DIP) and phenylarsine oxide, which would decrease the ROS, did not alter the I_{CaL} at basal or with hypoxia (117). These contrary results might be due to species differences or the type of ROS used. Nevertheless, it is now clear that ROS like H_2O_2 and HOCl do regulate the I_{CaL} and may increase or decrease the currents.

It is believed that ROS regulate I_{CaL} by directly reacting with, and thus altering the conformation of the L-type Ca^{+2} channels. Indeed, the pore-forming subunit α1C contains more than 48 cysteine residues that can potentially undergo redox modification (169). Cysteine residues in the β-subunit may also be important for the trafficking of the L-type Ca^{+2} channels (48). Some of these cysteines make disulfide bonds while others are free and are available for redox modification so that direct ROS-induced modification of the subunits of the L-type Ca^{+2} channels may affect the conducting properties, and thus modulate the I_{CaL}. As mentioned earlier, the results of ROS effects on I_{CaL} have been controversial, and the results with the application of oxidizing sulfhydryl (SH) agents are also contradictory in several studies. Nevertheless, it evident that I_{CaL} is modulated by various oxidizing agents. For

example, thiol-oxidizing agents such as thimerosal decreased I_{CaL}, through the human α1C-subunit expressed in HEK293 cells (76), through the rabbit cardiac Ca^{+2} channels expressed in HEK293 cells (116), and in isolated guinea pig ventricular myocytes (150). This inhibition of I_{CaL} in guinea pig myocytes is reversible by the reducing agent such as dithiothreitol (DTT) (150). Treatment of the canine myocytes with an oxidizing agent, buthionine sulfoxime, also attenuated I_{CaL} while this was reversed by treatment with GSH (42). On the contrary, several SH reagents induced I_{CaL} in frog ventricular myocytes, and this effect was independent of cAMP production or G-protein stimulation, indicating that ROS increased the I_{CaL} by directly modulating the Ca^{+2} channels (276). The effect of such oxidizing SH agents is also reversed by reducing agents such as GSH or DTT (276). Similarly, cGMP-independent direct application of NO causes S-nitrosylation of extracellular SH groups of the L-type Ca^{+2} channel, and therefore increases I_{CaL} (41). Regarding the above-mentioned studies, it is important to mention that the reducing agents or the cellular antioxidant CAT is ineffective on I_{CaL} by themselves under basal conditions.

These contradictory results might depend on the type, amount, and exposure time to ROS. For example, application of oxidizing agents such as 3-morpholinosydnonimine-HCl (SIN) to the ferret myocytes may cause an increase or a decrease in I_{CaL} (41) depending on the dominant mechanism of action. The myocytes in which SIN inhibited I_{CaL} the cellular redox conditions were such that the direct effects of NO were dominant, whereas in those myocytes in which SIN stimulated I_{CaL}, the indirect effects of $NO/O2^{\bullet -}$, which S-nitrosylate and/or oxidize signaling molecules, were dominant (41). Thus, the type, mechanism, and amount of ROS determined the I_{CaL} response. Similarly, in frog myocytes, methanethiosulfonate ethylammonium (MTSE) caused a biphasic increase and then decrease in I_{CaL} (276). Since MTSE penetrates the cell membrane very slowly, it is hypothesized that the MTSE perfusion of the whole cell alters the intracellular redox balance and causes an initial increase in I_{CaL}. After this initial increase in I_{CaL}, MTSE reaches in the cell in time and causes a decrease in I_{CaL} due to direct modulation of the channel proteins (276).

It is evident that direct application of ROS such as H_2O_2, HOCl, or several thiol-reducing or oxidizing compounds can alter channel function, but the response may vary. As previously mentioned, ROS may act by two different mechanisms, that is, (i) by direct modification of the channel proteins and (ii) by affecting the cellular signaling that in turn may affect the I_{CaL}. In fact, ROS affect the L-type Ca^{+2} channels activity *via* PKC (45) (Fig. 7). The mechanism of the effect of ROS-induced PKC on the activity of cardiac L-type Ca^{+2} channels is not exactly known; however, it is established that the α1C- and β2-subunits of cardiac L-type Ca^{+2} channel can be phosphorylated by PKC *in vitro* (279). In fact, various isoforms of PKC (α, β1, β2, and ε) are detected in both neonatal and adult ventricular myocytes (36, 279). The effect of PKC on I_{CaL} can be highly diverse. PKC can either decrease I_{CaL} (228, 292), and may even cause an increase and decrease in I_{CaL} during the same experiment in a biphasic response (151, 279). It is postulated that PKC phosphorylates the N′ terminus of the α1C-subunit of the L-type Ca^{+2} channel, and the effect on the channel can be either stimulating or suppressive (279). Recently, it was observed that the oxidation product of arachidonic acid, 20-hydroxyeicosatetraenoic acid (20-HETE), increases I_{CaL} in a concentration-dependent manner (289). This effect of 20-HETE was mediated by the decreased activity of NADPH oxidase that increased the cellular ROS, and thus PKC activity (289) (Fig. 7). In addition, ROS-mediated increase in sensitivity of the I_{CaL} to α-adrenergic receptor stimulation involves PKA. Since the activity of PKA can also be altered by ROS, PKA may be an important regulator of cell functions during changes in redox state (86, 119) (Fig. 7). In another study, angiotensin II increased I_{CaL} through an increase in the α1C-subunit mRNA and protein levels in rat atrial myocytes. This increase in the α1C occurred due to serine-133 phosphorylation of CRE-binding protein (CREB) by PKC, NADPH oxidase, and the ROS pathway (251) (Fig. 7). This indicates that apart from the short-term effects of ROS, in a long term, ROS can also alter the protein expression to affect the I_{CaL}. The ROS effects on I_{CaL} become more complicated by the fact that a number of different splice variants of L-type Ca^{+2} are expressed in the heart that may have variable sensitivity to ROS (77).

Although often contradictory, the results mentioned above allow some insight into how ROS influence L-type Ca^{+2} channel function and the mechanisms for the calcium-mediated changes in the action potential, excitability, and arrhythmia in cardiac myocytes. For example, the gain-of-function mutations G406R and G402S cause reduced Cav1.2 channel inactivation, resulting in maintained depolarizing I_{CaL}, prolongation of action potentials, and DADs (240). In another example, Mahajan *et al.* showed that I_{CaL} can be targeted to increase dynamic wave stability by maintaining action potentials without depressing contractility (172). Mahajan *et al.* showed that in rabbit ventricular myocytes, when I_{CaL} inactivation is inhibited/delayed, and when these myocytes were also treated with verapamil (an I_{CaL} blocker), the intracellular Ca^{+2} transients were not affected; the action potential duration restitution slope was flattened; and action potential duration alternans (beat-to-beat alternation in action potential duration) were prevented (172). In fact, this approach has been proposed as the possible therapeutic way to increase the usability/safety of I_{CaL} blockers as antiarrhythmic drugs. On the other hand, ROS-mediated decrease in I_{CaL} and thus a decrease in Ca^{+2} influx during the plateau phase of the action potential may be necessary to prevent arrhythmias associated with prolongation of the QT interval during hypoxia. However, hypoxia and direct application of ROS also decrease the sensitivity of the L-type Ca^{+2} channel to the α-adrenergic receptor agonist isoproterenol, and this may be deleterious to the cell (115). Despite these studies, it is not clear if ROS cause increase/decrease in I_{CaL} and how important this effect would be in arrhythmia biology. Nevertheless, for the most part, it has been observed that ROS cause a net increase in the cellular Ca^{+2} (91). This net increase in cytosolic Ca^{+2} is due to ROS affecting on other Ca-handling proteins as RYR, NCX, and SERCA, in addition to an increased/decreased I_{CaL}.

B. Effects of ROS on the RyRs

Ca^{+2} release from the SR through RyR channels is essential for activation of cardiac and skeletal muscle contraction. Three isoforms of RyRs have been identified. The RyR1 isoform is dominant in skeletal muscles, whereas the RyR2 is predominantly expressed in the cardiac cells (79). At the

amino acid level, the three mammalian RyR isoforms share 70% identity (79). The RyR has multiple sites for regulation, including cytosolic and luminal sites for regulation by Ca^{+2}, and cytosolic sites for Mg^{2+}, ATP, CaM, and FK-506-binding proteins (FKBP) (79).

The radical $O_2^{\bullet -}$ increases Ca^{+2} efflux from the reconstituted RyR from the heavy fraction of cardiac SR vesicles due to decreased CaM content (134). In addition, $OH^{\bullet}$ chemically generated by the reaction of H_2O_2 and Cu–ethylenediamine or the thiol-oxidizing agents also increased the open probability of the reconstituted channels formed by the cardiac RyR purified from the pig heart, and this effect was reversed by DTT (9). These results were supported by experiments in a native system of rat cardiomyocytes in which ROS production from mitochondria in intact rat cardiac myocytes was achieved by photostimulation and antimycin-A treatment (63, 278). Intracellular ROS and Ca^{+2} signals were simultaneously measured in fluorescence-based assays. Photoactivated or antimycin A-induced mitochondrial ROS production elicited a transient increase in Ca^{+2}-spark activity from RyR (63, 278). Lowering basal mitochondrial ROS production, scavenging baseline ROS, and applying DTT diminished the spontaneous Ca^{+2} spark activities and abolished the Ca^{+2} spark responses to mitochondrial ROS (63, 278). Such findings suggest that $OH^{\bullet}$ reacts with RyR and increases the Ca^{+2} release from the SR through the RyR.

The tetrameric RyR contains up to 89 cysteine residues per monomer (274). However, only a small number of hyperactive SH groups on the channel protein appears to have a redox-sensing function (274). Figure 8 shows the summary of the ROS effect on the functions of RyR directly or indirectly *via* other ancillary proteins. Oxidizing agents such as 2,2′-dihydropyridine (DTDP) or 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) activate the RyR channel (isolated from various species), and this effect was reversed by reducing agents such as DTT (67, 286). It has also been shown that H_2O_2 modulates the gating properties to increase the open probability of cardiac RyRs, and this effect was reversed by DTT (9, 208). In contrast, addition of NADH, which would increase $O_2^{\bullet -}$, decreased the RyR activity that decreased the frequency and the amplitude of spontaneous Ca^{+2} sparks, and this inhibitory effect was reversed by NAD^+ (299). However, this might be due to the direct inhibitory effects of NADH on the RyR independent of the mitochondrial function (299). Regarding the effect of endogenously generated $O_2^{\bullet -}$, it has been shown that exercise and tachycardia increase NADPH oxidase activity, which increases the cellular $O_2^{\bullet -}$ content, and thus enhanced the RyR activity (227) due to enhanced *S*-glutathionylation. The redox modulation of the RyR can also lead to alteration of the sensitivity of the channel to cytosolic Ca^{2+} and ATP. Redox modification of cysteine residues, for example by GSH/GSSG, alters the interaction of the RyR with triadin or CaM (19, 162). Triadin is a transmembrane ancillary protein that regulates the sensitivity of the RyR to luminal Ca^{+2} by stabilizing the binding of calsequestrin (an intra-SR Ca^{+2}-binding protein) to the RyR (97), and oxidation/reduction of the thiols on RyR alters the interaction with triadin, and thus the sensitivity to the luminal Ca^{+2}. Thus, oxidation of the SH groups in the triadin-binding site of RyR reduces triadin binding and may increase RyR activity (162). Similarly, oxidation of the RyR CaM-binding site by GSSG reduces the interaction with the CaM, and thus increases the RyR activity (19). Further information on regulation of RyR by CaM-CaMKII can be find in a review (173).

In support of these results, altered ROS content in acquired diseases is also important in determining the intracellular Ca^{+2} *via* RyR activity. For example, in isolated intact and permeabilized ventricular myocytes from a canine model of heart failure, the ratio of GSH/GSSG as well as the level of free thiols on RyR decreased markedly, consistent with increased oxidative stress in heart failure. With this increased ROS, RyR-mediated SR Ca^{+2} leak was significantly enhanced in permeabilized myocytes from the heart failure compared to control cells, and this was reversed with reducing agents such as DTT (93). In dystrophin KO cardiomyocytes, slow elevations of the intracellular Ca^{+2} resulted in Ca^{+2} oscillations with an enhanced Ca^{+2} sensitivity. This is due to the elevated cellular ROS generation in dystrophy that causes the abnormal RyR sensitivity to increases in the intracellular Ca^{+2} concentration (131), which can be arrhythmogenic. Similarly, after ventricular fibrillation, the myocytes from a canine model had an increased rate of ROS production and increased

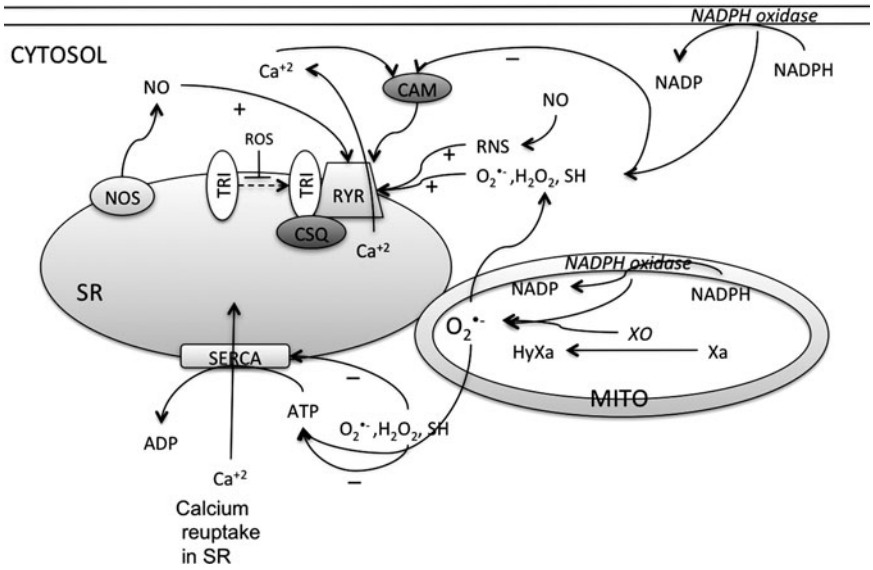

FIG. 8. RyR activity is enhanced, and SERCA pump activity is decreased by ROS/RNS. ROS/RNS enhance the RyR activity directly or by inhibiting the interaction with triadin (TRI) that alters the Ca^{+2} sensitivity of receptor in the SR. Triadin stabilizes binding of calsequestrin (CSQ) to the RyR. ROS also decrease the ability of RyR to bind with calmodulin (CAM), thereby decreasing the activity. ROS decrease the activity of SERCA by direct interaction or by decreasing the cellular ATP content necessary for the SERCA activity. XO, xanthine oxidase; HyXa, hypoxanthine; Xa, xanthine.

RyR oxidation (21). Abnormal RyR function in ventricular fibrillation cells was indicated by increased fractional Ca^{+2} release for a given amplitude of Ca^{+2} current and elevated diastolic RyR-mediated SR Ca^{+2} leak. Treatment of ventricular fibrillation myocytes with reducing agents normalized the parameters of Ca^{+2} handling and shifted the threshold of Ca^{+2} alternans to higher frequencies (21).

Because Ca^{+2} release by RyR depends on the intraluminal SR Ca^{+2} content, a prolonged ROS activation of RyR would exhaust SR Ca^{+2} content, and thus decreases RyR activity. The evidence that RyR dysfunction depends on the concentration and the length of exposure to ROS and may be biphasic is now briefly reviewed. Experiments on single-ventricular myocytes indicate that H_2O_2 causes Ca^{+2} overload as a result of activation of Ca^{+2} release from intracellular stores (and not from activation of voltage-dependent Ca^{+2} channels or NCX) (89), and after an initial stimulation of release, prolonged exposure to $O_2^{\bullet -}$ caused a decrease in the Ca^{+2} release by RyRs probably due to the depletion of SR Ca^{+2}. Similar biphasic responses were obtained when ROS synthesis in rat cardiomyocytes was induced by photoactivation or treatment with antimycin A (278). These treatments caused a transient increase in Ca^{+2} spark activity, followed by gradual spark suppression in phase with the mitochondrial ROS oscillations (278). Partial deletion of Ca^{+2} stores in the SR contributed in part to the gradual, but not the phasic, spark depression. H_2O_2 at 200 m*M* also elicited a bidirectional effect on sparks (278).

The mechanisms of NO action on the gating properties of RyR remain controversial. Nitrosylation of SH groups of cardiac RyR by low-molecular-weight S-nitrosothiols, such as or S-nitrosoglutathione (GSNO) and S-nitrocysteine (CysNO), stimulates channel activity (242). In NOS1-deficient mice, RyR hyponitrosylation leads to diastolic Ca^{+2} leak and a proarrhythmic phenotype (92). In addition, peroxynitrite ($ONOO^-$) generated by SIN (3-morpholinosydnonimine) activates the channel several fold through thiol oxidation (274). On the other hand, NO produced endogenously by NOS localized to the cardiac SR decreases the open probability of the RyR (287). It seems that effects of NO or NO-related molecules also depend on the concentration and cytosolic environment. Low concentrations of NO donors have been shown to activate the RyR, whereas high concentrations cause inhibition of the channel (104). From these studies, it is evident that the nitrosylation of RyR enhances the activity. Together, these findings demonstrated that a nitrosoredox imbalance causes RyR oxidation, hyponitrosylation, and SR Ca^{+2} leak, a hallmark of cardiac dysfunction (93).

In summary, based on the studies, we speculate that increased cellular ROS/RNS content oxidizes the cysteine thiols of the RyR, which may decrease its interaction with the regulatory proteins CaM and triadin, or may alter the RyR conformation. This would increase the open probability of the RyR causing release of SR Ca^{+2}, and thus increase the intracellular Ca^{+2} content. Thus, ROS-induced RyR activation might be associated with the depolarization of the membrane potential, prolongation of the action potential, and loss of excitability, to cause arrhythmia.

C. SERCA is affected by ROS

Because Ca^{+2} transport into the SR is tightly coupled to hydrolysis of ATP, inhibition of ATPase activity will ultimately decrease the Ca^{+2} pumping rate. Out of the three SERCA isoforms, SERCA1, SERCA2a and 2b, and SERCA3, SERCA2a is expressed in cardiac muscle. Redox agents are known to modulate SERCA (Fig. 8). The cardiac SERCA contains 25 cysteine residues (193). Reagents that oxidize thiols inhibit pump activity (opposite to the effect on RyR), whereas reducing agents (*e.g.*, DTT and GSH) protect SERCA from this inhibition (229). H_2O_2 also inhibits the ATP-dependent Ca^{+2} uptake activity in the microsomes from HEK-293 cells overexpressing SERCA2b (96), perhaps by directly interfering with the ATP-binding site (273). In a rat cardiomyocyte system, short-term H_2O_2 application induced SR Ca^{+2} depletion *via* redox-mediated decreased activity of SERCA and increased activity of the NCX, and this was associated with thiol oxidation (149). NO does not appear to affect SERCA Ca^{+2} pump activity directly; however, nitroxyl (HNO; the 1-electron-reduced and protonated form of NO) increased the maximal rate of Ca^{+2} uptake mediated by SERCA due to a reversible oxidative modification of SERCA thiols (2). HNO increased the *S*-glutathiolation of SERCA, and adenoviral overexpression of glutaredoxin-1 prevented both the HNO-stimulated oxidative modification of SERCA and its activation, as did overexpression of a mutated SERCA in which cysteine 674 was replaced with serine. Thus, HNO increases the maximal activation of SERCA *via S*-glutathiolation at cysteine 674 (2, 153). More information on oxidative modulation came from G-alpha-q- (alpha-subunit of Gq protein) overexpressing mice (152). The isolated ventricular myocytes from G-alpha-q mice had marked abnormalities of myocyte calcium transients along with an increased oxidative stress. Increased ROS in these G-alpha-q myocardia caused irreversible sulfonylation at cysteine 674 and nitration at tyrosines 294/295 of SERCA protein without affecting the total expression (152). Due to these oxidative modulations of SERCA, the calcium-stimulated SERCA activity was decreased. Crossbreeding G-alpha-q mice with transgenic mice that have cardiac myocyte-specific overexpression of CAT decreased SERCA oxidative cysteine modifications, decreased SERCA cysteine-674 sulfonylation and tyrosine-294/295 nitration, restored SERCA activity, and improved myocyte calcium transients and contractile function (152). Thus, in contrast to the effects on the RyR, ROS/RNS decreased the activity of the SERCA and reduced the Ca^{+2} reuptake into the SR. This may increase the total Ca^{+2} and decreased Ca^{2+} release in the myocytes, causing prolongation of the action potential and thus decreased excitability. Further information on SERCA regulation by CaMKII can be found in a review (173).

VI. Redox and Potassium Channels and Excitability

Cardiac K^+ currents can be distinguished on the basis of differences in their functional and pharmacological properties. In mammalian cardiac cells, K^+ channels can be categorized as voltage-gated (K_v) and ligand-gated K^+ channels (155) (Table 2). In addition, in ventricular myocytes, it has been shown that the inhibition of leak K^+ currents is sufficient to delay repolarization, causing prolongation of the action potential duration, and thus affects the excitability of the cells (24). Although the molecular identity of this large group of K-leak channels has been identified, their exact functions in cardiac cells, number, types, and effects of various intracellular signaling molecules, including ROS, are not very well known. Thus,

Table 2. Various K^+ Currents in the Cardiac Myocytes and Their Associated K^+ Channel Subunits

Class	*Current*	*α-subunit*	*Gene*	*β-subunit*	*Gene*
Kv channels	I_{Kto}	Kv4.3	KCND3	KChIP2	KCNIP2
		Kv1.4	KCNA4		
		Kv4.1	KCND1	KChIP1	KCNIP1
		Kv4.2	KCND2	KChIP2	KCNIP2
	I_{KUR}	Kv1.5	KCNA5	Kvβ1	KCNAB1
				Kvβ2	KCNAB2
	I_{Kr}	Kv11.1 (HERG)	KCNH2	minK	KCNE1
				MiRP1	KCNE2
	I_{Ks}	Kv7.1	KCNQ1	minK	KCNE1
	I_{K1}	Kir2.1, Kir2.2	KCNJ2, KCNJ12		
	I_{KCa}	K_{Ca}1.1 (BK_{Ca})	KCNMA1		KCNMB
		K_{Ca}2.1 (SK_{Ca}1)	KCNN1	CaM	CALM
		K_{Ca}2.2 (SK_{Ca}2)	KCNN2	CaM	CALM
		K_{Ca}2.3 (SK_{Ca}3)	KCNN3	CaM	CALM
		K_{Ca}3.1 (IK_{Ca})	KCNN4	CaM	CALM
Ligand Gated K^+ channels	I_{KACH}	Kir3.1, Kir3.4	KCNJ3, KCNJ5		
	I_{KATP}	Kir6.2, Kir6.1	KCNJ11, KCNJ8	SUR2A, SUR2B	ABCC9
Leak K^+ channels	K-Leak currents	K_{2P} channels—TWIK, TREK, TASK, TALK, THIK, and TRESKSubfamilies	KCNK1,2,3,4,5,6,7,9, 10,12,13,15,16,17,18		

The K^+ channels are made up of an α-subunit and a β-subunit. The proteins and genes associated that make these channels are tabulated. Voltage-gated K-channels open in response to a change in the membrane potential to permit transmembrane passage of K^+ according to inherent voltage- and time-dependent properties. The Kv channels include the rapidly activating and inactivating transient outward current (I_{Kto}), the ultrarapid (I_{Kur}), rapid (I_{Kr}), and slow (I_{Ks}) components of the delayed rectifier and the inward rectifier (I_{K1}), whereas the ligand-gated K^+ channels include those that respond to a decrease in the intracellular concentration of ATP (K_{ATP}) or activated by ACH (K_{Ach}) (155). In addition, K^+ channels that are activated by intracellular Ca^{2+} are called calcium-activated K^+ channels (K_{Ca} channels). These channels are difficult to classify because of their structural dissimilarity with the other Kv channels, but because their activation follows opening of the voltage-dependent Ca^{2+} channels, functionally they have been classified as voltage-sensitive K^+ channels. In addition, K-leak channels have also been defined in the excitable cardiac cells, which conduct voltage-independent ligand-independent leak K^+ currents (247). CaM, calmodulin. The table was created by adapting the information from (24, 33, 245, 247, 285).

BK_{Ca}, large-conductance K_{Ca}; SK_{Ca}, small-conductance K_{Ca}; IK_{Ca}, intermediate-conductance K_{Ca}; KChIP, K^+ channel-interacting protein; SUR, sulfonylurea receptors.

these channels will not be reviewed further. The molecular constituents of various K^+ channels are also shown in Table 2. K^+ channels are responsible for the repolarization of the action potential, and thus any functional modulation of these K^+ channels can disrupt excitation. Delay or inhomogeneity of the repolarization may initiate arrhythmias by causing EADs and TA or re-entrant arrhythmias, respectively. Different types of K^+ channels are differently modified by ROS, an observation that might be of importance in disease states. ROS interaction and their effect on various K^+ channel activities are reviewed below.

A. Effects of ROS on the Kv channels

At least five major K^+ currents (I_{Kto}, I_{Kur}, I_{Kr}, I_{Ks}, and I_{K1}) flowing through Kv channels possess different density and voltage- and time-dependent properties that contribute to repolarization in the ventricular myocardium (245) (see Fig. 9). While I_{Kto} causes early repolarization of the action potential plateau leading to a notch in the action potential shape, activation of the delayed rectifier currents, I_{Kur}, I_{Kr}, and I_{Ks}, together with a decrease in I_{Ca}, controls final repolarization. The late phase of the final repolarization is further supported by I_{K1} (245). ROS affect all the Kv channels as shown in Figure 9. ROS affect the activation and the inactivation kinetics of various I_{Kv}, and thus the net result of ROS depends on primarily whether the activation kinetics or the inactivation kinetics was affected. For example, a ROS-induced enhanced inactivation or slower activation will lead to more channels in the open state, and thus an increased I_{Kv}. Nevertheless, most of the studies have demonstrated that ROS decrease cardiac repolarizing K^+ currents, which prolong the action potential, and thus prolong the effective refractory period, due to which the propensity of EAD increases (181, 211, 247). It is noteworthy that some of the studies have also demonstrated unaffected I_{Kto} or I_{K1} by direct application of oxidants as t-BHP or H_2O_2 (27, 263). The reasons for these contradictory results are unclear, but could presumably be direct effects of these oxidants on other channels/systems of the cell.

Several inward and outward K^+ channels are affected by different ROS-generating systems. For example, in guinea pig cardiac ventricular myocytes, ROS generated from the xanthine/xanthine oxidase system or H_2O_2 decreased the inward K^+ current (53, 199). In a comprehensive study, Duprat *et al.* (66) reported that when expressed in *Xenopus* oocytes, the activity of three Shaker K^+ channels (Kv1.3, Kv1.4, and Kv1.5), one Shaw channel (Kv3.4), and one Kir channel (IRK3) was drastically inhibited by photoactivation of rose bengal, a classical generator of ROS. On the other hand, Shaker K^+ channel Kv1.2; Shab channels Kv2.1 and Kv2.2; Shal channel Kv4.1; and inward rectifiers IRK1 and ROMK1, and hIsK were completely resistant to this treatment. In contrast, t-BHP, another generator of ROS, removed the fast inactivation processes of Kv1.4 and Kv3.4, but did not alter other channels. In *Xenopus* oocytes, external application of H_2O_2 was found to

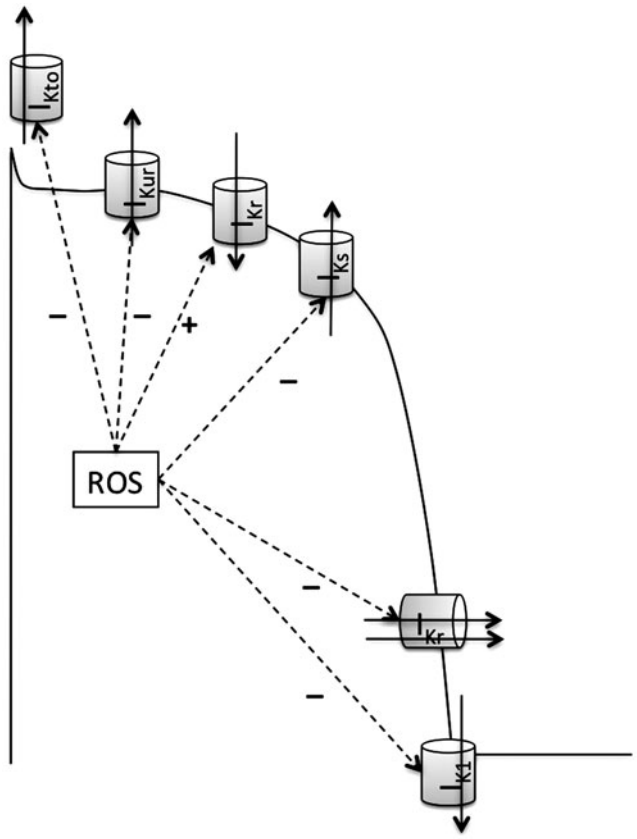

FIG. 9. ROS modulate the activity of various voltage-dependent K^+ currents that contribute to the cardiac action potential. Various K^+ currents such as the transient outward current (I_{Kto}), the ultrarapid (I_{Kur}), rapid (I_{Kr}), and slow (I_{Ks}) components of the delayed rectifier and the inward rectifier (I_{K1}) are shown on the action potential when their activity is maximum. The outward currents I_{Kto}, I_{Kur}, I_{Ks}, and I_{Kr} and the inward I_{K1} are all inhibited, whereas the outward current by HERG, a depolarizing phase, is generally increased by ROS.

inhibit the time-dependent fast inactivation process of three cloned voltage-gated K^+ channels, KShIIIC, KShIIID, and HukII (256), in a reversible manner. According to the authors, H_2O_2 caused a large increase in the current magnitude because of the inactivation block due to which some channels would still be opening while a significant fraction of channels would have been already inactivated. Other cloned voltage-gated K^+ channels were not affected by H_2O_2 (256). Furthermore, transient nonlethal ionizing irradiation of the whole cell or H_2O_2 or heat stress induced whole-cell voltage-dependent outward K^+ currents with no changes in the membrane potential of -70 mV, intracellular K^+ concentration, or ATP levels (148). Similarly, Kv7.1-coded I_{Ks} currents were not modulated in CHO cells (84), but in the human submucosal gland serous cell line Calu-3, Kv7.1-coded I_{Ks} currents were increased by H_2O_2 perfusion (58). All of these studies were done in the heterologous system with exogenous expression of the subunits of the K^+ channels and have provided complex results, suggesting that ROS may increase the activity of certain Kv channels while it may decrease activity of other Kv channels.

More comprehensive results came from the studies in native systems using isolated ventricular myocytes. For example, in isolated guinea pig ventricular myocytes, the ROS-generating system DHF reduced the outward K^+ current (44) (Figs. 9 and 10). The effect of ROS on I_{Kto} [measured as a difference between peak current (I_{peak}) and steady state current (I_{ss})] was demonstrated (160) in a native system. Exposure of rat ventricular myocytes to a thio-specific oxidant, diamide, decreased the I_{peak} and I_{ss} (measured at the end of the test pulse) by about 50% after depolarizing test pulses. The decrease in I_{peak}, but not the decrease of I_{ss}, was reversed by inhibiting the thioredoxin system (160). The ROS-induced decrease in I_{ss} was reversed by inhibiting the glutaredoxin systems (Fig. 10). From this study, it might be inferred that the thioredoxin system controls the Kv4.2, Kv4.3, or Kv1.4 channels that constitute the I_{peak} (see Table 1). Since Kv1.5 and Kv2.1 (I_{Kur} and I_{K1}) channels largely contribute to I_{ss} in rat myocytes, it may be postulated that one or both are regulated by the glutaredoxin system. In another study with the left ventricular myocytes of rats in which redox pathways were inhibited, GSH content was reduced; ROS were increased; and the maximum I_{Kto} density was reduced, whereas the I_{K1} and I_{Ks} (Kv7.1) were not significantly altered (159) (Fig. 10). Decreased I_{Kto} density may be due to ROS-induced decrease in mRNA and proteins levels of Kv4.2 and Kv1.4, which was reversed *in vitro* by exogenous GSH or N-acetylcysteine (a GSH precursor and antioxidant). This was also shown in the myocytes in which the increased oxidative stress decreases the mRNA and protein expression of Kv4.2 and Kv4.3 channel alpha-subunits as well as the accessory protein KChIP2 (159). Thus, results from the experiments in the native system suggest that ROS cause a decrease in the Kv currents (I_{Kv}; I_{Kto}, I_{Kur}, I_{Kr}, I_{Ks}, and I_{K1}) in myocytes. One of the deadly dysfunctions of Kv current is Long-QT syndrome, in particular LQT1 (estimated 35% of LQT patients), which is caused by decreased I_{Ks}. The decrease in I_{Ks} decreases repolarizing currents, causing severe prolongation of the action potential (QT interval), and a reduced refractory period, both of which will render the myocytes unexcitable and will generate arrhythmia (28).

The crucial role played by Kv channels in the control of cell excitability has gained considerable attention after discovering that the LQT2 was caused by mutations in the human ether-a-gogo-related gene (*HERG*) or *KCNH2* encoding the channel Kv11.1 responsible for I_{Kr} (59). The main features of I_{Kr} are its modulation by the extracellular concentration of K^+ ions, a peculiar inward rectification mechanism at depolarizing potentials, and its blockade by class III antiarrhythmics (230). However, at negative voltages, the channels recover from the inactivation faster than they deactivate, leading to more open channels and significant outward current (230) (see Fig. 11). When *Xenopus* oocytes, expressing Kv11.1, were exposed to an extracellular solution containing $FeSO_4$ and ascorbic acid (Fe/Asc) (as a cellular ROS production system), outward I_{Kr} carried by Kv11.1 channels was increased, whereas inward currents were not modified (244). Currents carried by other K^+ channels such as Kir, bEAG, rDRK1, and mIRK1 remained unaltered. This ROS-induced increase in outward I_{Kr} carried by Kv11.1 was due to a depolarizing shift of the voltage dependence of channel inactivation, with no change in channel activation (244). This effect would increase the availability of the channels to be opened in response to

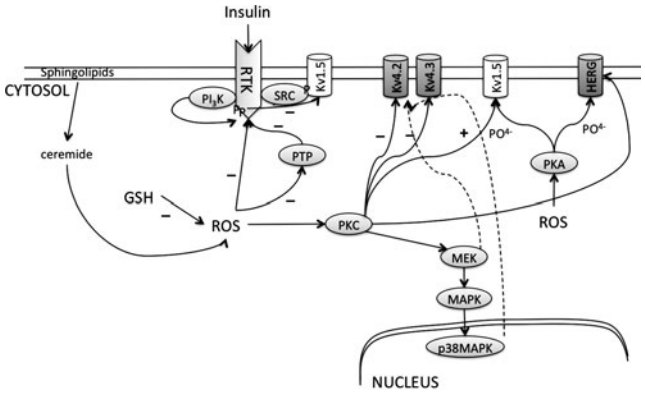

FIG. 10. ROS effects on the voltage-gated K^+ channels. Phosphorylation of Kv1.5, Kv4.2, Kv4.3, or HERG decreases the currents from these channels. ROS activate PKC or PKA that are responsible for this phosphorylation. ROS also inactivate protein tyrosine phosphatase (PTP), causing increased phosphorylation of the receptor tyrosine kinases (RTK) that leads to activation of SRC-mediated phosphorylation of Kv1.5. ROS content is increased by cytosolic fatty acids such as ceramide and decreased by GSH.

depolarization. At negative potentials, the channels are fully recovered from inactivation, and therefore the modulation by Fe/Asc of the inactivation process cannot be exerted, possibly explaining the lack of Fe/Asc-induced modulation of the inward current component of Kv11.1 channels (244). Fe/Asc-induced stimulation of Kv11.1 outward currents was completely prevented by scavenging ROS with a mixture of CAT, SOD, and mannitol (244). In the similar experiment, H_2O_2 enhanced the Kv11.1 outward currents by accelerating the activation at depolarizing voltages, whereas decreased the inward currents by accelerating the deactivation at repolarizing voltages in the CHO cells expressing human Kv11.1 channels (23). Effects of H_2O_2 superfusion were prevented by intracellular application of CAT, but SOD prevented only H_2O_2-induced acceleration of activating currents (23). Contrary to these results, hypoxia in a stimulus- and time-dependent manner increased ROS and decreased Kv11.1 protein with a marked reduction in Kv11.1 K^+ conductance in

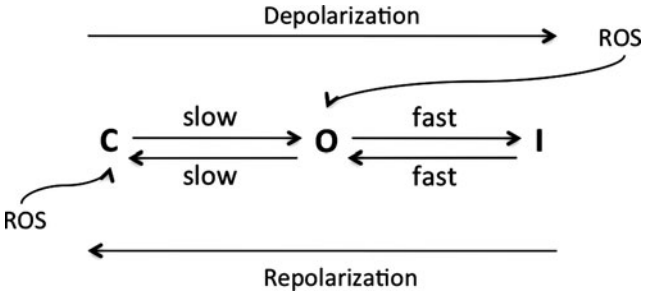

FIG. 11. Schematic of activation–inactivation kinetic model for HERG. ROS cause a depolarization shift in inactivation kinetics, and thus leads to more open channels to increase outward currents. At repolarizing potentials, most of the channels recover from inactivation to be active. However, ROS accelerate the deactivation at the repolarizing voltages, decreasing the inward currents. C, closed-deactivated; O, open; I, inactivation.

HEK cells stably expressing the Kv11.1 α-subunit (202). Importantly, this downregulation of Kv11.1 by hypoxia was not due to increased proteasomal degradation or decreased transcription, but due to decreased synthesis of the protein (202). Antioxidants prevented hypoxia-evoked downregulation of Kv11.1 protein and exogenous oxidants (xanthine/xanthine oxidase system) that mimicked the effects of hypoxia, and yet the inhibitors of NADPH oxidase failed to prevent the effects of hypoxia (202). This study was somewhat confusing, because in this study, hypoxia increased ROS instead of decreasing, and thus the actual mechanism by which hypoxia modulated Kv11.1 currents is not clear.

It is important to define whether ROS regulation of Kv channels occurs by direct thiol modification of Kv subunits or by indirect effects on redox-sensitive signaling molecules. In this regard, phorbol 12-myristate 13-acetate (PMA), a nonspecific activator of serine/threonine and tyrosine protein kinases, or phenylepherine, suppresses Kv4.2 and Kv4.3 currents in a heterologous system, and native I_{Kto} in rat ventricular myocytes, in a PKC-dependent manner (198) (Fig. 10). PKC decreases these currents by increasing the inactivation time constants and slows the time course of recovery from inactivation of I_{Kto} (198). Since cellular ROS activate PKC (47), increased ROS will reduce the I_{Kto} in a PKC-dependent manner. ROS cause transient oxidation of thiols in protein tyrosine phosphatases that leads to their inactivation, and this activates receptor tyrosine kinases (RTK) (47). Activation of RTK causes phosphorylation of the Kv channels (exemplified by Kv1.5 and Kv1.3) *via* SRC kinase that represses Kv currents (112). In this regard, diabetic rat ventricles showed decreased I_{Kto} due to a decreased GSH content. However, upon activation of RTK by insulin in these myocytes, GSH levels and I_{Kto} were restored (158). This was further confirmed in the myocytes from diabetic rats that were treated for several hours with GSH or NAC, to increase the cellular antioxidant capacity, and this treatment restored the I_{Kto} density (158). Therefore, ROS could also reduce the I_{Kto} and I_{KUR} by indirectly activating the tyrosine kinase (receptor-mediated or nonreceptor-mediated, such as SRC). Overall, it can be conceived that increased ROS will decrease I_{Kto} and I_{KUR}, whereas decreased cellular ROS would increase the I_{Kto} and I_{KUR}. Furthermore, GSH upregulation due to activation of RTK by insulin was also blocked by inhibitors of PI_3-kinase, MEK, and p38 MAP kinases (158), indicating that modulating these kinases could also decrease I_{Kto}. Human Kv1.5 (I_{KUR}) also has one consensus site for phosphorylation by PKC located on the extracellular S4–S5 linker and 4 consensus sites for PKA located in the N- and C-terminal domains. Therefore, both PKC and PKA activation inhibited I_{KUR} (156). Since PKC and PKA are stimulated by ROS, ROS might regulate I_{KUR} in humans as well. PKA and PKC might also be involved in the Kv11.1 I_{Kr} regulation, because ceramide, a sphingolipid metabolite, depresses I_{Kr} mainly *via* ROS overproduction, and this suppression was reversed by PKA and PKC inhibitors, but not by tyrosine kinase inhibitors (17). These PKC and PKA effects occur presumably by direct phosphorylation of the Kv11.1. However, there are other modes by which ROS cause direct alteration of the Kv11.1 channels, and thus reduce Kv11.1 currents, which depend on the molecular makeup of the Kv11.1 itself. To localize the molecular regions involved in ROS-induced modulation of Kv11.1, segmental exchanges between the ROS-sensitive Kv11.1 and the ROS-insensitive

bovine ether-a-gogo gene (bEAG) channels have been generated, and the sensitivity of these chimeric channels to ROS was studied (213). Results obtained suggest that histidines at positions 578 and 587 in the S5–S6 linker region of Kv11.1 channels are important in ROS-induced modulation of Kv11.1 channels (213). Moreover, mutagenesis revealed that cysteine 723, a conserved residue in a structural element linking the C-terminal domain to the channel's gate, is critical for ROS modification (141). For the most part, it is confirmed that the expression and activity of cardiac I_{Kv} are regulated by endogenous ROS, *via* receptor tyrosine kinase/PKC signaling, which functionally impacts K^+ channel. However, effects of ROS on every K^+ channel type and the functional impacts of ROS-induced alterations in cell signaling need further study. In summary, it seems like increased cellular ROS decreased the voltage-dependent K^+ currents in the myocytes. This will reduce the total repolarizing currents that may lead to a depolarized membrane potential, an enhanced action potential duration, and decreased excitability.

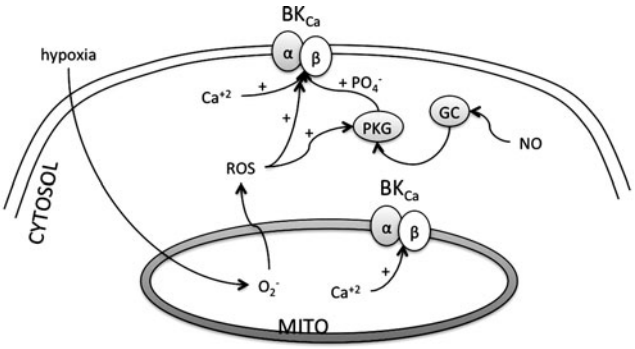

FIG. 12. ROS enhance the activity of the large-conductance calcium-activated K-channels (BK_{Ca}). Activation by ROS occurs due to increase in cytosolic Ca^{+2}, as well as directly by modulating channel subunits. Nitric oxide (NO) activates protein kinase G (PKG) that increases phosphorylation of the BK_{Ca} channels to increase the activity.

B. Calcium-activated K^+ channels

Calcium-activated K (K_{Ca}) channels are a key signaling target in smooth muscle cells, opening of which causes hyperpolarization. The three types of K_{Ca} channels, large conductance K_{Ca} (BK_{Ca}), the small conductance K_{Ca} (SK_{Ca}), and the intermediate conductance K_{Ca} (IK_{Ca}), might have a different expression pattern in various tissues (245). The BK_{Ca} channels are expressed in the adult and neonatal chick ventricular myocytes and conduct K^+ currents (125). The function of the cardiac BK_{Ca} is debatable, because the α-subunit gene of the BK_{Ca} is not expressed in cardiomyocytes of certain species as rodent; however, BK_{Ca} channels have been reported in the guinea pig cardiomyocyte inner mitochondrial membrane, where it plays a cardioprotective role (118). SK_{Ca} channels are also expressed in human and mouse cardiac myocytes; however, SK_{Ca} channels were more abundant in the atria and pace-making tissues compared with the ventricles (252). All of the three isoforms of SK_{Ca} channel subunits (SK1, SK2, and SK3) are expressed. It has been shown that SK_{Ca} channels contribute functionally toward the shape and duration of cardiac action potentials (157). Therefore, genetic KO of the SK2 channels results in the delay in cardiac repolarization and atrial arrhythmias (157).

The effects of ROS on the activity of K_{Ca} have not been studied in detail; however, it has been shown that hypoxia causes repression of the BK_{Ca} β-subunit expression in cardiac mitochondria in a Hif-2α-mediated mechanism and prevents deleterious mitochondrial depolarization (38) (Fig. 12). Whether this hypoxia effect causes any decrease in BK_{Ca} currents and if the mechanisms involve ROS is unclear. In this regard, H_2S has been shown to reduce the conductance from the native BK_{Ca} channel in rat glomus cells and from the human recombinant BK_{Ca} channel in HEK293 cells (248). In another study, NO stimulated the activation of BK_{Ca} channels in *Xenopus* oocytes, in which the α- and β-subunits of the human BK_{Ca} channels were expressed along with a receptor GC and rat atrial natriuretic peptide receptor (204). Site-directed mutation of consensus phosphorylation sites on the β-subunit (Ser855 and Ser869 to alanine) indicated that NO causes augmentation of the BK_{Ca} currents *via* GC-mediated stimulation of PKG, which phosphorylates the residues 855 and 869 (204). From these studies, it is clear that ROS/RNS do modulate the BK_{Ca} currents, but not by a direct redox modulation of the channel subunits. The effects of ROS on cardiac SK and IK channels need further investigation.

C. K_{ATP} channels

K_{ATP} channels are found at a high density in the heart (210). The cardiac K_{ATP} channels are formed by a K_{ir} pore (6.1 or 6.2) and the sulfonylurea receptors (SURs) that act as the regulatory subunits (210). Expression of Kir6.1, Kir6.2, and the three isoforms of SUR viz SUR2A, SUR2B, and SUR1 has been demonstrated in the cardiac sarcolemma as well as the mitochondrial membranes (7, 154, 210). Moreover, it has been demonstrated indirectly by pharmacological techniques that active functional K_{ATP} channels do exist in both sarcolemma as well as mitochondria membranes (87). Despite ample evidence of K_{ATP} activity in sarcolemmal membranes (sarc-K_{ATP}) and mitochondrial membranes (mito-K_{ATP}), direct molecular evidence of the existence of the mito-K_{ATP} is weak (210). It is generally recognized that the predominant form of K_{ATP} channels in cardiac sarcolemma is K_{ir}6.2-SUR2A (7). The identity of mitochondrial K_{ATP} channels has been a subject of investigation for years with variable results, suggesting that mito-K_{ATP} could be Kir6.1-SUR2A, Kir6.2-SUR2B, or other heteromeric combination of these subunits (7). This has been further complicated by the expression of various splice variants of the SURs recently discovered in the mitochondrial membranes (282).

Since the opening and K^+ conductance of the K_{ATP} channel depends on the cellular ATP, the activity of the channels is directly correlated to the metabolic status of the cell and the availability of the O_2 (Fig. 13). Therefore, K_{ATP} channels are also generally accepted as the metabolic sensors of the cell that play a major role in protecting the heart from ischemia–reperfusion-induced damage (87). The activation of sarc-K_{ATP} channels reduces the action potential duration (decreased excitability) and conserves energy during periods of ischemia (30). The activation of the mito-K_{ATP} channel increases the K^+ influx in the mitochondria, depolarizes the mitochondrial membrane potential, increases the Ca^{+2} tolerance of the mitochondria, and conserves the structural integrity (87).

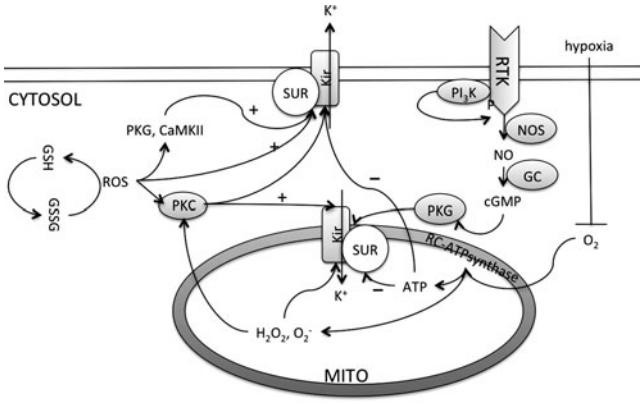

FIG. 13. ROS activate K_{ATP} channels. K_{ATP} are formed by sulfonylurea receptors (SURs) and inward rectifier (Kir) on sarcolemma or mitochondrial membranes. ROS-mediated activation of Protein Kinase-C and G (PKC and PKG) activates K_{ATP}. ROS, such as H_2O_2, itself activate K_{ATP} directly. Hypoxia decreases mitochondrial ATP synthesis and thus cellular ATP content, which lead to increased opening of mitochondrial or sarcolemmal K_{ATP}, respectively.

However, this increase in potassium conductance due to the opening of sarc-K_{ATP} channels may predisposes heart to electrical dysfunction, and in some cases, the generation of fatal arrhythmia (30). Therefore, the opening of sarc-K_{ATP} channels, such as in an ischemic heart, can significantly shorten the action potential and promote arrhythmia, and if enough channels open, can render cells unexcitable (26, 109). These reductions in the action potential duration are exacerbated by K_{ATP} channel agonists (68, 122) and blocked by the K_{ATP} inhibitors such as glibenclamide, HMR 1833, or HMR 1098 (69, 81, 223). Various K_{ATP} inhibitors also reduce the incidence of postischemic ventricular arrhythmia in rat, rabbit, pig, dog, and in humans (15, 80, 254, 265); however, this blockade of the sarc-K_{ATP} causes increase in the infarct sizes of heart postischemia (95).

Since hypoxia and the metabolic state of the cell regulate ROS generation, K_{ATP} channels are affected by ROS directly or indirectly *via* signaling. In fact, the first K^+ channel that was found to be modulated by hypoxia and ROS in the heart was the K_{ATP} channel when hypoxia, presumably at a level able to cause metabolic inhibition, was shown to activate a K^+ conductance that was called K_{ATP} (206). About 15 min of hypoxia increased the I_{KATP}, whereas GSH could reverse the increased I_{KATP} (123, 250, 277). In this regard, in the isolated myocytes, GSSG increased the I_{KATP}, while GSH could reverse the increased I_{KATP} during normoxia. The redox couple DTT/H_2O_2 produced results similar to the redox couple GSH/GSSG during normoxia (277). H_2O_2 increased the I_{KATP} in a concentration-dependent manner, which was reversed by DTT or glibenclamide (123). H_2O_2 at concentrations of 5 m*M*, but not 1 m*M*, increased K_{ATP} channel activity in the presence of 0.3 m*M* ATP in ventricular cells isolated from guinea pig hearts. However, the presence of 10 μM ADP and Mg^{2+} together with 0.3 m*M* ATP increased the sensitivity to H_2O_2 and led to activation of the K_{ATP} channel by 1 m*M* H_2O_2 (123). The channel activation was due to an increase in the open-state probability and was not associated with a change in the single-channel conductance or the mean open and closed times. In addition, the ATP sensitivity of the channels was also decreased by 1 m*M* H_2O_2; therefore, H_2O_2 activates the K_{ATP} channel directly by decreasing the sensitivity to ATP, an effect potentiated by the presence of ADP (26, 123). $OH^{\bullet}$ produced *via* the iron-catalyzed Haber–Weiss reaction in the guinea pig ventricular myocytes also activated K_{ATP} channels; however, this activation could not be prevented by even very high concentrations of glibenclamide (250). Several experiments have induced ventricular arrhythmias under normoxic conditions with delivery of ROS bursts, and scavenging the ROS with SOD mimetics, or mitochondrial-targeted antioxidant peptides were successful in decreasing the incidence of arrhythmia (49, 105, 143). The cellular mechanisms by which ROS cause K_{ATP} activation are not clear; however, it is believed that increased ROS deplete cellular ATP that causes K_{ATP} channels to open. Nevertheless, the I_{KATP} augmented by hypoxia and GSSG was reversed by inhibition of the PKC by bisindolylmaleimide VI, inhibition of PKG by KT5823, and inhibition of CaMKII by KN-62 and KN-93 (277). Inhibition of PKA by H-89 did not reverse the hypoxia or GSSG effect (277) (Fig. 13). These results suggest that the effects of both GSSG and hypoxia on K_{ATP} channels involve the activation of the PKC, PKG, and CaMKII pathways, but not the PKA pathway. Whether there is also a direct modification of the K_{ATP} channels by the redox is unclear.

It has been observed that under conditions of metabolic stress, sarc-K_{ATP} currents and action potential durations oscillate in cardiac myocytes (12, 224). These fluctuations in sarc-K_{ATP} currents and, consequently, the action potential duration are intricately linked to the $\Delta\Psi_m$ (12, 224). The $\Delta\Psi_m$ collapses with an increased ROS generation in the intact myocyte or isolated mitochondria from various species. However, this collapse in $\Delta\Psi_m$ is reversible, and thus oscillates, in isolated cells or the heart subjected to oxidative stress *via* modulating GSH/GSSG or NAD/NADH, ATP depletion, local generation of ROS by laser excitation, chemical oxidants, and respiratory inhibition, or to global ischemia/reperfusion (10, 12, 222, 224, 234). With this oscillatory $\Delta\Psi_m$, sarc-K_{ATP} current increases or decreases in phase with the $\Delta\Psi_m$, which may alter the action potential, and thus excitability, by altering K^+ currents (Fig. 14). The exact mechanism of this $\Delta\Psi_m$-induced arrhythmia is not known yet, but has been postulated to be due to the local generation of mitochondrial ROS that induce further ROS generation by neighboring mitochondria in a spaciotemporal network (40). This term is coined as "ROS-induced ROS release." The increase in ROS under conditions of oxidative stress can reach a critical level, after which cell-wide $\Delta\Psi_m$ oscillations in the mitochondrial network are observed (12) (Fig. 15). Indeed, in isolated cardiac myocytes, oxidants caused oscillations in the $\Delta\Psi_m$, which were accompanied by ventricular tachycardia/fibrillation, but was reversed by GSH addition (10, 234). With all these lines of evidence, there are very few studies that have focused on the role of mitochondria and mito-K_{ATP} in regulating the $\Delta\Psi_m$ and generation of arrhythmia. The $\Delta\Psi_m$ and the mitochondrial ROS generation have been correlated to the increased K^+ flux in the mitochondria. Although the identity of the mito-K_{ATP} is still under investigation, it is widely accepted that such a K^+ flux across the mitochondrial membrane occurs due to a mito-K_{ATP} channel that opens upon metabolic inhibition and causes collapse of the $\Delta\Psi_m$ with concomitant ROS generation (56, 87). In this regard, direct measurement of

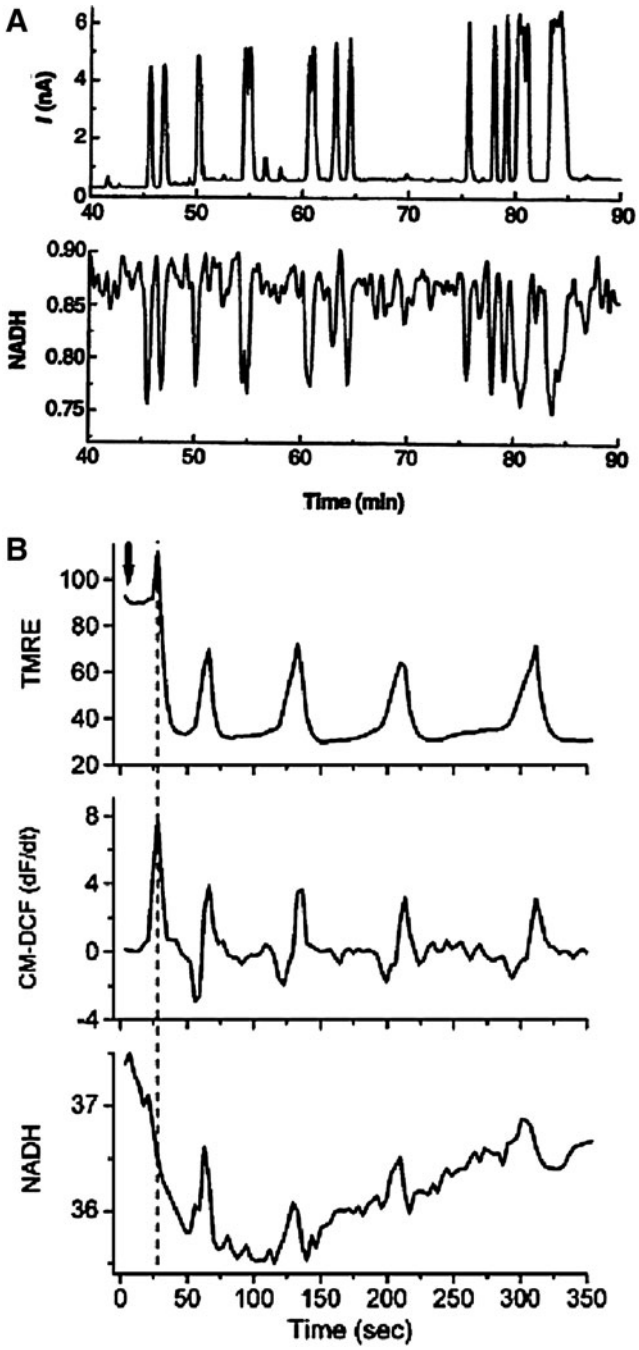

FIG. 14. Membrane excitability and mitochondrial energetics. (A) In-phase oscillations of the sarc-K_{ATP} currents correlate with the oscillations of the NADPH fluorescence in adult guinea pig myocytes. The NADPH fluorescence in shown in uncalibrated units, and the membrane currents were measured at −40 mV. This indicated that mitochondrial functions might be related to the cellular excitability. **(B)** Simultaneous, whole-cell recordings of the temporal evolution of the fluorescence intensity of TMRE (mito-membrane potential-sensitive), CM-DCF (ROS-sensitive), and the endogenous NADH signals (ROS-sensitive). Compounding these results, it has been suggested that the mitochondrial ROS production and mito-membrane potential influence the membrane sarc-K_{ATP} currents and thus membrane potential. Panels for this figure are taken from (54, 207), with copyright permission.

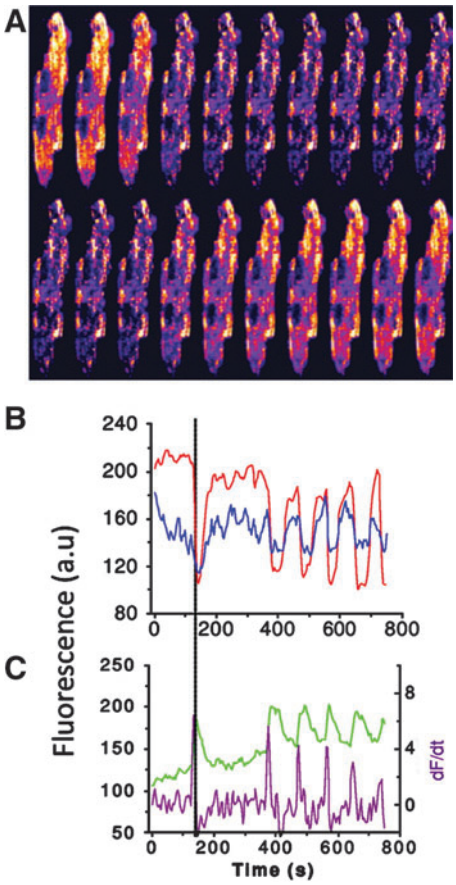

FIG. 15. Characterization of cell-wide mitochondrial oscillations. (A) Images of a cardiomyocyte loaded with TMRE (for detection of mitochondrial membrane potential) and DCF (for detection of ROS generation at the frame interval of 7 s. The phasic increase in intensity of fluorescence is evident. **(B)** Time course of average whole-cell fluorescence of TMRE and NADH (as an indicator of ROS), and **(C)** DCF and the derivative of DCF signals (dF/dt, purple). The precise phase relationship between all signals can be clearly appreciated from the vertical reference line drawn in **(B, C)**. The individual panels in the figure were originally published in Aon *et al.* (11) © The American Society for Biochemistry and Molecular Biology. Reprinted with permission.

the mito-I_{KATP} in fused mitoplasts or bilayer mitochondrial membranes has been demonstrated successfully (124, 127). Thus, it is evident that the ROS activate mito-K_{ATP}, which enhances K^+ flux in to the mitochondria, depolarizes the mitochondria, and inhibits the respiratory chain. This causes even more increase in the ROS generation in presence of the trace amount of O_2. Whether and how ROS actually open mito-K_{ATP} will remain a speculation until the identity of the mito-K_{ATP} has been confirmed. However, it has been observed that activation of PI3-kinase leads to phosphorylation of Akt and eNOS, which induced NO synthesis, which stimulates GC to produce cGMP, which activates PKG, ultimately leading to opening of mito-K_{ATP} in the mitochondrial inner membrane (55). Although the role of mito-K_{ATP} in protection against the I/R injury and postischemic arrhythmia has been widely studied by various groups (56, 87, 95), more studies are needed to understand the role of mito-K_{ATP} toward action potential, excitability, and arrhythmia.

VII. Redox Effects on Sodium Channels and Excitability

The cardiac Na^+ channel is a member of the voltage-dependent family of Na^+ channels. These channels consist of heteromeric assemblies of an α-subunit that forms the pore and ancillary β-subunits that modulate the function (25). The human cardiac Na^+ channel α-subunit is a heavily glycosylated protein of ~260 kDa consisting of 2016 amino acid residues encoded by the gene *SCN5A*, whereas the human β1-subunit is a 218-amino-acid protein encoded by *SCN1B* (88, 175). Several other isoforms of the α-subunit (Nav1.1–Nav1.3) encoded by *SCN1A–SCN3A* and β-subunit (β1, β2, β3 and β4.1, and β4.2) encoded by *SCN1B, SCN2B, SCN3A,* and *SCN4A* have also been described in the cardiomyocytes. The functions of various β-subunits in the heart are unclear and controversial in the literature. Investigations have revealed several functions of the β-subunits, including the increase in expression of α-subunits ($Na_v1.5$) in the sarcolemma, augmentation of the amplitude of I_{Na}, modulation of the gating properties, and regulating the interaction of $Na_v1.5$ proteins to extracellular matrix molecules, cytoplasmic cytoskeleton apparatus, and components of cardiac intercellular junctions (*e.g.*, cadherins and connexins) (8). Regarding the existence of several subunit isoforms, studies have reported controversial results on the constitution of ventricular Na^+ channels. Na^+ channels composed of $Na_v1.5$ and β2- and/or β4-subunits have been shown in intercalated disks of the myocytes (174), and channels composed of $Na_v1.1$, $Na_v1.3$, and $Na_v1.6$ with β1- and/or β3-subunits have been shown in the transverse tubules (174). On the contrary, $Na_v1.1$ or $Na_v1.5$ with β1- and β2-subunits has been shown at the Z-line. Yet, the β2-extracellular domain did not modulate the functions of the $Na_v1.5$ in a heterologous system. A β2 KO mice showed reduced neuronal Na^+ currents, but effects on cardiac Na^+ currents were not studied (46). β1 KO mice showed severely longer QT and RR intervals compared with the controls (165). Electrophysiological investigation in ventricular myocytes isolated from β1 KO mice revealed increased densities of both I_{Na} and I_{NaP} with no other changes in channel gating. A longer action potential duration and a slower rate of action potential repolarization were also found, consistent with the QT prolongation phenotype and the increase in I_{Na}. (165). The finding that the absence of β1 increased I_{Na} density contrasts with most *in vitro* reports, which show an increase of I_{Na} in the presence of β1. These discrepancies may be explained by the fact that the physiological heterologous expression systems do not fully recapitulate the situation in cardiac myocytes.

Confirming the key role of (-subunits in cardiac Na^+ channel regulation, a novel mis-sense mutation (L179F) in *SCN4B*, the gene encoding the (β4-subunit, was recently identified in a 21-month-old girl affected by Long-QT syndrome (LQT10) (25). Patch-clamp studies in HEK293 cells stably expressing Nav1.5 and transfected with this mutant β4-subunit revealed increased I_{NaL} compared to Nav1.5 expressed alone or together with the wild-type β4-subunit (185). Although the functional biology of the (-subunit is not clear, these studies do indicate that functional defects in Na^+ channel (-subunits are associated with arrhythmogenecity.

Despite the unclear and unestablished roles of β-subunits, it is clear that both the α- and β-subunits are highly expressed in the human heart (25), and that both the α- and β-subunits when expressed together in the heterologous system conduct larger currents, show accelerated activation and inactivation, and a more negative voltage dependence of steady-state inactivation compared with the α-subunit alone (25). The voltage-gated cardiac Na^+ channel is responsible for the generation of the rapid upstroke of the action potential, and thereby plays a central role in excitability of myocardial cells. In addition, these channels also determine the velocity of the upstroke and the impulse conduction velocity in cardiac tissue, and therefore are also important for the impulse propagation. Mutations in the gene encoding this channel (*SCN5A* and *SCN1B*) cause mainly three forms of primary electrical disease—the LQTS, the Brugada syndrome, and cardiac conduction defects (18, 25, 231). Ample studies have demonstrated that the ROS-induced alterations in I_{Na} electrophysiology in cardiac myocytes are responsible for various arrhythmogenic conditions by affecting the action potential and excitability (237, 238, 263).

Na^+ channels transit among various conformational states in the process of voltage-dependent gating. Depolarization from the resting membrane potential triggers activation (opening) of the Na^+ channels. If the depolarization is maintained, the channels enter a nonconducting inactivated state. Subsequent to repolarization, the channels return to a closed state capable of being activated once again (18, 231). Therefore, I_{Na} increases rapidly on depolarization, reaching a transient peak lasting a few milliseconds (I_{NaT}), before beginning to inactivate. The inactivation of I_{Na} has a fast component (fast inactivation) that lasts a couple of milliseconds and is followed by a slowly inactivating component that can last hundreds of milliseconds (18, 25, 231). The current associated with the slow inactivating component has been referred to as late, sustained, or persistent I_{Na} (I_{NaL}, I_{Na}sus, or I_{NaP}) to distinguish it from the peak transient I_{NaT}.

In a recent report, it has been demonstrated that elevated mitochondrial ROS generation, due to increased cytosolic NADH/NAD ratio, reduces I_{Na} in HEK cells stably expressing human cardiac Na^+ channel subunits (163). The effect of ROS on Na^+ channels is summarized in Figure 16. ROS, such as H_2O_2, increases I_{NaP} and thereby cause (i) prolongation of APD, and thus induction of EADs that decreases excitability; and (ii) a rise in intracellular Na^+ that causes cellular Ca^{+2} overload due to Na^+/Ca^{+2} exchange (237, 238, 263). As mentioned in previous sections, this Ca^{+2} overload in cardiomyocytes is associated with electrical instability and *via* affecting I_{Na}. Whole-cell and cell-attached patch-clamp techniques on isolated guinea pig ventricular myocytes showed that ROS regulate the I_{NaP} and I_{NaT}. Hypoxia for 15 min or application of GSSG increased I_{NaP} and decreased I_{NaT} at the same time, and this effect on I_{NaP} and I_{NaT} was reversed by GSH. Therefore, hypoxia for 15 min [here it was assumed that hypoxia increases ROS as in (300)] and GSSG decreased the action potential amplitude and shortened the action potential duration at 90% repolarization (APD_{90}) of ventricular papillary cells simultaneously, while GSH could reverse the decreased amplitude and the shortened APD_{90} at the same time (262). It is not clear from this study how hypoxia causes increase in ROS, the effect of which could be reversed by antioxidants. It can be assumed that hypoxia caused an actual decrease in ROS (and not increase) and that decreases the propensity of the Na^+ channels to inactivate or to remain inactivated causing an increased I_{NaP}. However, it is still not clear from this study how an increase in I_{NaP} could

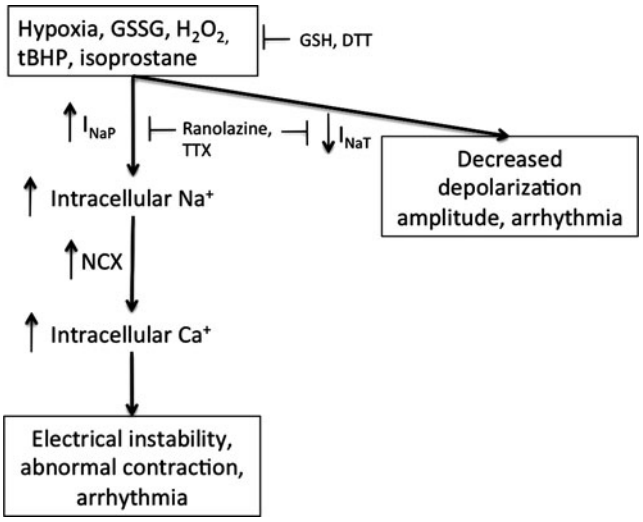

FIG. 16. ROS enhance persistent sodium currents (I_{NaP}), but decrease transient Na^+ currents (I_{NaT}). Increased I_{NaP} increases cytosolic Na, which then increases the cytosolic Ca^{+2} *via* increase Na/Ca exchange by NCX. Decreased I_{NaT} decreases depolarization amplitude and may cause arrhythmia.

cause shortened action potential duration. Wang *et al.* (262) did mention that both the currents might be charged with the same channel with a different gating mode. Thus, when I_{NaP} increases, the I_{NaT} might decrease and vice versa. The net effect of this interplay between I_{NaP} and I_{NaT} after hypoxia causes action potential duration shortening. Here it should be noted that in wild-type Na^+ channels, this noninactivating current component (I_{NaP}) is negligibly low (<0.3% of the transient current), but in the LQT3-mutant channels (where the action potential duration is prolonged), this fraction is increased (261). Regarding hypoxia and induction of I_{NaP} by hypoxia, drugs that are more-potent inhibitors of I_{NaP} have been in development. For example, ranolazine, flacainide, and mexiletin, which reduce I_{NaP} more potently than I_{NaT}, have been in study for the arrhythmias (due to increased I_{NaP}) caused by ischemia (*i.e.*, hypoxia) (226). In another study, milrinone (a phosphodiesterase-III inhibitor) was shown to reduce the I_{NaP} induced by hypoxia and H_2O_2 (293). Milrinone also reversed the prolonged APD_{90} (action potential duration and 90% repolarization) due to hypoxia and H_2O_2 in guinea pig myocytes (293). Similarly, other studies have shown that H_2O_2 can cause increases of I_{NaP} (167, 237, 238, 263) and intracellular Na^+ and Ca^{2+} (258), thereby increasing APD and thus the excitability. Therefore, ranolazine and tetrodotoxin (TTX) effectively reduced H_2O_2-induced I_{NaP}, action potential prolongation and EADs in isolated myocytes. Other oxidants such as t-BHP reduced I_{NaT}, by enhancing inactivation in HEK cells (which expressed cardiac Na^+ channels) or myocytes (83) mimicking the effects of hypoxia and GSSG on I_{NaT}. Products of the cellular lipid peroxidation isoprostanes that are markers and the mediators of the oxidative stress mimicked the effects of t-BHP on Na^+ channel function in HEK cells (83). In this regard, another reactive product of lipid peroxidation, 4-hydroxynonenal, was ineffective on the I_{NaT}, suggesting that physiologically generated ROS products do have a specific pathway by which they may or may not modulate I_{NaT} (83). In HEK cells stably expressing the cardiac Na^+ channel or in cardiomyocytes, cytosolic NADH application (to increase mitochondrial ROS generation) caused the decrease of I_{Na}, and this effect was reversed by mitochondrial antioxidants or by inhibiting the mitochondrial respiratory chain that would inhibit the ROS generation (163).

Although the mechanism is not clear, the results have been consistent showing that I_{NaP} and I_{NaT} in cardiac muscle can be modulated and regulated by ROS/RNS and other oxidants as well as with hypoxia. However, this is difficult to reconcile, as hypoxia is associated with a decreased synthesis of superoxide. It is possible that the increased production of NO reported during hypoxia/ischemia (137) contributes to the increase in I_{NaP} during hypoxia. Indeed, like hypoxia, exogenous NO also increases I_{NaP} in isolated hippocampal neurons (99) and in guinea pig myocytes (168), and these effects were abolished by GSH and DTT (3, 99, 168). These experiments suggest that NO acts by oxidizing the cardiac Na^+ channel protein components that regulate I_{NaP}, or a closely associated regulatory protein, contained within the plasma membrane. On the contrary, another report showed that NO decreases the I_{Na} in guinea pig and mouse cardiomyocytes without affecting the activation inactivation or reactivation kinetics. Instead, NO decreases open probability and/or decreases the number of functional channels with no change in single-channel conductance (4). Additionally, application of cell-permeable analogs of cGMP or cAMP mimics the inhibitory effects of NO, and that these effects cannot be reversed by a sulfhydryl-reducing agent (4). This report suggests that the NO affects I_{Na} by the cGMP/cAMP pathway and not by direct modulation of the Na^+ channels. In essence, NO and its RNS do alter I_{Na} through multiple pathways, and the results (although controversial) may alter the cellular excitability.

The fact that ROS/RNS-mediated I_{Na} can be reversed by the reducing agents suggests that the modulated Na^+ channel activity occurs as a result of a ROS/RNS-induced covalent modification or disulfide bond formation at or near the channel protein, although the mechanism underlying is unclear. It is known that there are 42 cysteine residues in the α-subunit of the human cardiac Na^+ channel (18). They distribute in the four S5–S6 linkers, intracellular linkers between domains I and II, II and III, and other regions of the channel (18). Thus H_2O_2, GSH/GSSG, DTT, and perhaps hypoxia could change the gating mode of the sodium channel by directly acting on one or more cysteine residues in the sodium channel (166). In this regard, NO and perhaps the RNS are shown to decrease the I_{NaP} and I_{NaT} possibly by S-nitrosylation of the cysteine residues on the α-subunit of the Na^+ channel (3, 253). It is not clear if and how nitrosylation occurs, but inhibitors of NOS could prevent the increase in I_{NaP} and I_{NaT}. It is interesting to note that the Na^+ channel coimmunoprecipitates with nNOS (253), and the increase in the Na^+ channel activity is associated with an initial increase in the intracellular Ca^{+2}, which in turn depends on the activity of plasma membrane Ca^{+2} ATPase (PMCA), syntropin, and caveolii (3, 253). The effects of ROS on β-subunits have not been studied as far as we know. However, in one study, experimental heart failure in dogs caused increased I_{NaL} due to slower decay, and this effect was concluded to be due to dysfunctioning of the β1-subunits (186). Regarding this study, it is to note that HF causes increased ROS generation in the

myocytes and that might have caused the dysfunctioning of β-subunits. However, it was not clearly tested in this study or other studies possibly because the identity and function of the β-subunits in the myocytes are still unclear. There are several proteins that interact with the Na^+ channels to alter/modulate Na^+ channel functions. A latest review article (1) can be referred on this subject for a detailed update. In particular, signaling pathways that regulate cardiac Na^+ channel function include activation of PKA, PKC, and CaMKII (Fig. 16), both of which as activated by ROS (166). The PKA phosphorylates two serine residues (S525 and S528 in the human cardiac isoform) within the DI–DII linker that augments the I_{Na} (82), whereas PKC phosphorylates S1505 in the DIII–DIV inactivation gate (217) that causes a reduction in the current amplitude at hyperpolarized (−94 mV) holding potential. Not only that, PKC activation also causes a negative shift in the voltage-dependence of steady-state inactivation (217). Thus, ROS may cause reduction in I_{NaT} indirectly by activating PKC. Therefore, ROS decrease I_{NaT} while increasing I_{NaP} in a reversible manner by directly interacting with the Na^+ channel subunits or by activating kinases. This will cause in the prolongation of the action potential duration and increase in cellular Na^+ and Ca^{2+} levels, which will ultimately lead to the impaired cardiac excitability. More information on ROS-mediated increased Na^+ and Ca^{2+} levels in cardiomyocytes has been mentioned in section V. In short, increased intracellular Ca^{2+} content activates CaMKII that in turn activates Na^+ channels to increase I_{NaP}. From several, it is indicative that presumably ROS alter Na^+ channel activity *via* affecting various signaling molecules such as PKC. However, studies have not elucidated a clear mechanism.

It has been shown that ROS oxidize methionine 281/282 at the regulatory domain of CaMKII, and the oxidized CaMKII has properties equivalent to autophosphorylated CaMKII (73), which remains constitutively active to phosphorylate a number of proteins. It has also been shown that CaMKIIδ can phosphorylate cardiac Na^+ channels, leading to increased I_{NaP}, and the consequent cellular Na^+ and Ca^{2+} overload was significantly slowed in myocytes lacking CaMKIIδ (259). CaMKIIδ altered Na^+ channel gating to reduce availability at a high heart rate, while enhancing I_{NaP} that prolongs the action potential duration. More recently, it has been shown that ROS require CaMKIIδ to increase I_{NaP} that ultimately increased intracellular Na^+ and Ca^{2+} levels to cause arrhythmias (260). Conversely, an increase of I_{NaP} would be expected to cause elevation of intracellular Ca^{2+}, and thus activation of CaMKII. Recently, in rat ventricular myocytes, it has been shown that an increased Nav1.5-dependent I_{NaP} activates CaMKII, and this effect was absent in the $Na_v1.5$ KO mouse myocytes (280). These results suggest that the mechanism of increasing I_{NaP} by ROS is possibly a complex pathway that includes Ca^{2+} and several kinases (such as CaMKII and PKC) and a self-regulating ability. The increased activity of the I_{Na} that prolongs the influx of sodium and extends repolarization is also responsible for most of the LQT3 cases (185, 226).

VIII. Redox Effect on Other Ion Channels, Exchangers, and Pumps

A. IP3R receptors

Like the RyRs, there are three IP3R isoforms referred to as type-1, 2, and 3; however, only IP3R (type-2) is expressed in the heart (139). In ventricular, atrial, or Purkinje myocytes, immunofluorescence studies show that IP3Rs are found in the perinuclear region and in the nuclear membrane (121). The role of IP3R in cardiac excitation is controversial, because IP3R expression in the heart is very low compared to that of RyRs (139, 191). Therefore, majority of the intracellular Ca^{+2} release occurs through the RyR; however, Ca^{+2} release *via* IP3R in atrial and Purkinje myocytes has caught some attention (110, 161). In adult mammalian atrium, IP3-dependent Ca^{+2} release enhances atrial Ca^{+2} signaling and exerts a positive inotropic effect (297). In addition, by facilitating Ca^{+2} release, IP3R may also be an important component in the development of Ca^{+2}-mediated changes in action potential, excitability (*via* inducing CaMKII-mediated I_{Na} and I_K), and arrhythmias (297). Interestingly, IP3Rs, ankyrin-B, Na^+/K^+ ATPase, and NCX co-localized in cardiomyocyte T-tubules in discrete microdomains (190), and thus it is possible that the local Ca^{2+} release by IP3Rs causes activation of these channels to ultimately affect the action potential and excitability. Similarly, in Purkinje fiber cells, cell-wide Ca^{2+} waves have been suggested as a possible mechanism for increased excitability and spontaneous generation of action potential (37). Indeed, it has been demonstrated that activated IP3Rs promote the development of Ca^{2+} sparks and waves in Purkinje fiber cells that may be proarrhythmic (110). Evidence for redox modulation of the IP3R comes only from noncardiac tissue or in a heterologous system. For example, IP3Rs are downregulated when vascular smooth muscle cells are stimulated by H_2O_2 (177). This decrease in IP3R by H_2O_2 is accompanied by a reduction in calcium efflux induced by IP3 (177). Contrary to this, H_2O_2 has been shown to directly activate the IP3R in endothelial cells (294). In HeLa cells, the thiol reagent thimerosal increased the sensitivity of the IP3R to basal IP3 levels (129). In permeabilized hepatocytes, GSSG and t-BHP stimulate the IP3R by increasing the binding affinity of IP3 (71, 72, 107). These effects were also reversed by reducing agents such as DTT. In addition, endothelin-1 (ET-1) that activates IP3R also increases the ROS generation (78). Whether ET-1 induced IP3R and enhanced ROS generation are linked is not known (78). The effect of ROS on IP3R in cardiac myocytes needs further investigation, and if that has any influence on the cardiac excitability is to be determined.

B. Plasmalemmal Ca^{+2} ATPase

PMCA, like SERCA, is a member of P-type ATPases and is responsible for expulsion of Ca^{+2} from the cytosol of the cells. The PMCA pump isoforms, PMCA1 and PMCA2, are expressed in the heart, and are thought to specifically reduce Ca^{+2} in subsarcolemmal microdomains (243). However, PMCA activity is not significantly involved in cellular excitability, as the amount of Ca^{+2} extruded by this pump in adult myocytes is negligible (<1%) (243), and only PMCA4b has been suggested to regulate a membrane regional pool of Ca^{+2} involved in cardiomyocytes [as shown in the transgenic rats that overexpress PMCA4b in the heart (101, 266)]. However, it can be assumed that the lower expulsion of Ca^{2+} from the cell due to inactivity of PMCA could cause a localized subsarcolemmal increase in $[Ca^{2+}]_I$, which could affect various other channels to alter action potentials. ROS can substantially inhibit both Ca^{+2} transport and ATPase activity of PMCA presumably by decreasing the cellular ATP content in stressed

conditions, or by direct modulation of the channel protein. For example, in brain synaptosomes, peroxyl radicals, H_2O_2, and $ONOO^-$ decreased the PMCA activity. The effect of $ONOO^-$ was the most potent, followed by peroxyl radicals and H_2O_2. These effects of ROS are reversible, and occur through oxidation of SH groups and peroxidation of membrane phospholipids (288). A similar effect may occur in cardiac tissue, but more studies are needed.

C. Sarcolemmal and mitochondrial Na^+/Ca^{+2} exchanger

The Na^+/Ca^{+2} exchanger (NCX) is an antiporter membrane protein that removes Ca^{+2} from cells in exchange of Na^+ using the energy from ATP. The NCX removes a single Ca^{+2} ion in exchange for the import of three Na^+ ions (136), and the current carried with it (I_{NCX}) contributes to the late phase of the action potential (281). Membrane depolarization or high intracellular Na^+ can cause Ca^{+2} entry into the cell *via* reverse mode action of NCX, and thus NCX also serves important role in maintaining Na^+/Ca^{+2} homeostasis (136) and cellular excitability. Increase in the $[Na^+]_I$ or an increase in extracellular Ca^{2+} could cause an outward shift in I_{NCX} and thus a shortening of the action potential. On the other hand, increase in $[Ca^{2+}]_I$ may cause an inward shift in I_{NCX} and thus the prolongation of action potential. Thus, the activity of NCX is important for the generation and morphology of action potential and thus cellular excitability. Indeed in the canine myocytes, NCX repolarizes the action potential at high $[Na^+]_I$ (>10 m*M*), but contributes to the depolarization at low $[Na^+]_I$ (<5m*M*) (14). Cardiac myocytes express sarcolemmal NCX protein at high levels, and when it is upregulated/overexpressed, it mediates Ca^{+2} influx (instead of efflux) during the cardiac action potential (140). Cardiac NCX (encoded by the gene *NCX1*) consists of nine transmembrane domains, and intramolecular disulfide bonds between cysteine residues of different domains have been implicated to be functionally relevant (136). The activity of the cardiac NCX is sensitive to modification by ROS. Indeed, NCX activity is profoundly inhibited by hypoxia in guinea pig ventricular myocytes, but is reactivated within 1–2 min of reoxygenation possibly due to rising ROS (70). Similarly, H_2O_2 perfusion or ROS generation by XOR system induced NCX activity in rabbit ventricular myocytes (90). Whether or not these changes occurred due to direct modulation of NCX is unknown. However, a recent study showed that H_2O_2 increased the activity of NCX in adult rat ventricular myocytes (149) due to thiol modification. Furthermore, CAT overexpression increased NCX expression in cardiac myocytes (221), suggesting that NCX expression might have been decreased by ROS. Again, whether this is due to direct effects of ROS is unknown. Similarly, NCX activity in bovine cardiac sarcolemmal vesicles was stimulated up to 10-fold by preincubating the vesicles with $FeSO_4$ and DTT in an NaCl medium, which was then reversed by GSH (219). The combination of $FeSO_4$ and DTT removed Na^+-dependent inactivation of NCX and activated the exchanger, and this effect was reversed by CAT. Therefore, changes in the NCX activity depend on a rearrangement of disulfide bonds between cysteine residues that is modulated by the ROS or ROS scavengers. Because significant bursts of ROS generation occur during reperfusion of the ischemic heart, changes of NCX activity are likely to play an important role in ischemia-related Ca^{+2} overload.

Mitochondrial Ca^{+2} fluxes play a very important role in cell physiology. During cell activation, the increase in cytosolic Ca^{+2} triggers influx of Ca^{+2} in the mitochondrial matrix through a mitochondrial calcium uniporter, which is then released out of mitochondria by a mitochondrial NCX (34). The role of mitochondrial Ca^{+2} fluxes and mitochondrial NCX in the generation of arrhythmia is much less clear. It is known that H_2O_2 or the XOR system increased the mitochondrial Ca^{+2} concentration ($[Ca^{+2}]$m) at least in the endothelial cells, and that this raised $[Ca^{+2}]$m was secondary to the increases in the cytosolic Ca^{+2} increases (284), and due to inhibition of the NCX of the mitochondria (128). Similarly in intact hearts, perfusion with oxygen radicals resulted in a reduction in $[Ca^{+2}]$m, which was partly reversed by SOD and CAT (284). Further studies are need to elucidate if ROS affect NCX by a direct or an indirect mechanism and how that effects the cardiac excitability.

D. Sodium–potassium ATPase pump

The sodium–potassium ATPase (Na/K-ATPase) is an ATP-dependent ion pump and serves as a receptor for the cardiotonic steroids (CTS) like digoxin. The CTS inhibit the Na/K-ATPase, elevating $[Na^+]$i that in turn raises $[Ca^{2+}]$i *via* NCX. However, lower concentration of CTS (concentration that does not inhibit the Na/K-APTase) can stimulate the Na/K-ATPase to exert activating or deactivating effects on several signaling pathways in the myocytes. For details on these effects, the readers are encouraged to refer the review by Zhang *et al.* (291). Various pathways affected by the Na/K-ATPase include Src binding that makes a complex with epidermal growth factor receptor (EGFR) [Na/K-ATPase-Src-EGFR complex], the Ras/Raf/MEK/ERK1/2 pathway, mitochondrial ROS production, the activation of the IP_3 pathway that eventually leads to transcriptional activation of several proteins, and the activation of the PKC pathway (98, 142, 189, 269, 271). Thus, the functions of Na/K-ATPase are much more complex than just increasing the intracellular Ca^{2+} to promote contracture. In addition, it has been shown that factors such as PKC, Ca^{2+}, and ROS can modulate the activity of the Na/K-ATPase directly or through various signaling pathways. ROS directly induce the endocytosis of the Na/K-ATPase, thereby reducing its plasma membrane density, and hence its activity at the cell surface (270, 272). It is assumed that this occurs due to a direct interaction of ROS with the Na/K-ATPase pump to alter the conformation. Additionally, ROS affect the Na/K-ATPase pump indirectly *via* PKC, CaMKII, and intracellular Ca^{2+}. Ca^{2+}/CaM-dependent CaMKII activity phosphorylates the Na/K-ATPase and inhibits its activity (205). The effects of PKC on the Na/K-ATPase activity have been controversial. PKC has been shown to phosphorylate the Na/K-ATPase α1-subunits to promote the exocytosis of Na/K-ATPase molecules from the late endosomes into the sarcolemmal membrane, causing an increased Na/K-ATPase activity (103). On the other hand, PKC has also been shown to induce endocytosis of the Na/K-ATPase, thereby reducing its density in the plasma membranes, and hence Na/K-ATPase activity at the cell surface (209). The reasons to these controversial results are not clear, but it is indeed clear, and PKC regulates the activity of the Na/K-ATPase, which in turn is

regulated by ROS. Since the Na/K-ATPase pump is the primary regulator of the resting membrane potential (by exchange of $3Na^+$ to $2K^+$), it does regulate the excitability. ROS-induced decrease in Na/K-ATPase activity may prolong the refractory period, and thus may reduce cellular excitability. This may rise to EADs or DADs that will cause arrhythmia. An inactivated Na/K-ATPase (for example, by CTS) increases the cellular Na^+ that inhibits NCX activity, and thus retains cellular Ca^{2+} to increase cardiac contracture. However, it is noteworthy that ROS increase the activity of the NCX (as reviewed earlier). Therefore, it can be assumed that ROS could increase the efficacy of CTS (toxicity of CTS) by increasing cellular Na^+ and greater retention of Ca^{2+} due to a subsequent action of NCX. It is also important to consider that ROS increase the activity of RYR that will further increase $[Ca^{2+}]i$. By the action of ROS on the Na/K-ATPase, ROS will eventually accumulate cations in the cells that could cause the prolongation of the effective refractory periods and thus reduction in the excitability. However, increase in the total depolarizing currents may give rise to EADs that will propagate as arrhythmias. Thus, in presence of ROS, the same amount of CTS will cause toxicity and arrhythmogenesis. The effect of CTS and ROS on the Na/K-ATPase activity and RYR to cause arrhythmogenesis has been recently studied by Ho *et al.* (111) (here CTS were shown to increase ROS).

E. Mitochondrial inner membrane anion channel

Several distinct energy-dissipating ion channels in the inner membrane have been proposed to be involved in the $\Delta\Psi_m$ collapse, contributing to the generation of arrhythmia. One of these channels is the inner membrane anion channel (IMAC) (40). Although the identity of the IMAC is unknown, electrophysiologically, it is known that the IMAC is responsible for anionic currents across the mitochondrial membrane (12). Due to these currents, the IMAC regulates the $\Delta\Psi_m$, its reversible collapse, and thus membrane potential oscillations in cardiac myocytes (12). This indicates that targeting the IMAC may be effective in preventing arrhythmias by stopping ROS-induced ROS release. A confirmatory role for IMAC involvement in arrhythmia was provided in a series of studies where inhibiting the IMAC prevented arrhythmias in intact mammalian hearts (39). Whether or not ROS affect the IMAC function is not known partly, because the identity of the IMAC is unknown, and this needs further studies.

IX. Overall Effect of ROS on Action Potential, Excitability, and Arrhythmia

In summary, under pathological conditions, such as heart failure and ischemia–reperfusion, ROS levels can become elevated and predispose the heart to arrhythmias by altering the cardiac action potential. ROS induce functional impairment of various ion channels, including Na^+ channels (I_{NaT} and I_{NaP}), L-type Ca^{2+} current ($I_{Ca}L$), K^+ currents (I_{Kto}, I_{Kr}, and I_{KATP}), RyR, SERCA2a, and the NCX (Fig. 17 and Table 1). This impairment might be due to a direct modulation of the cysteine SH–SH on the channel subunits or indirectly by affecting the signaling molecules in the myocytes that may ultimately affect the functions of these ion channels. In ventricular myocytes, I_{Na}, $I_{Ca}L$, RYR, and NCX cause inward currents, with a net increase in intracellular Ca^{+2} and Na^+,

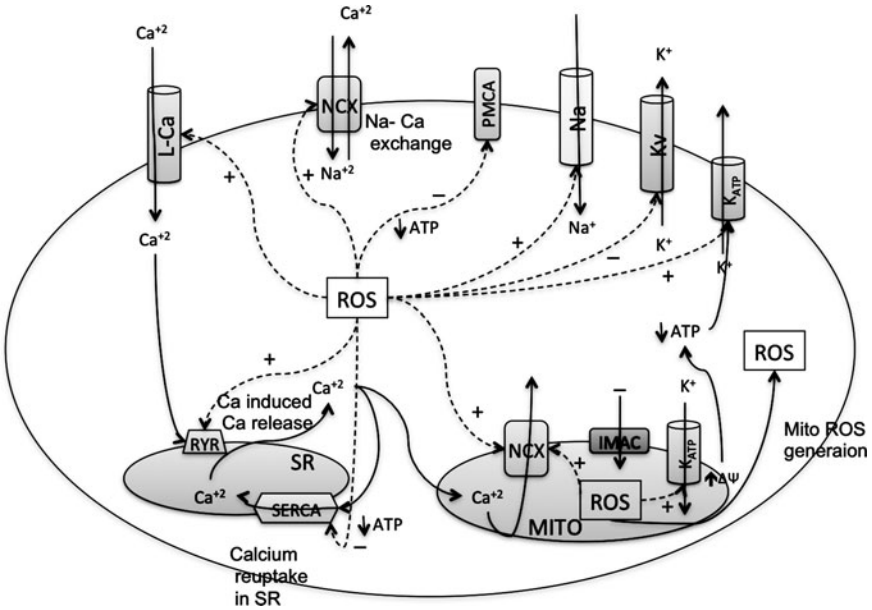

FIG. 17. Effects of ROS on cardiac ion channels, cytosolic ionic balance, and excitation-contraction coupling. ROS increase (although controversial) the activation of L-type Ca^{2+} channels, RyR, and mitochondrial NCX, but decrease the activity of SERCA and PMCA. This increases cytosolic Ca^{+2} content. Increased cytosolic Ca^{+2} is expelled out of the cytosol by ROS-mediated increased activity of NCX that increases cytosolic Na^+ content. ROS also increase Na^+ content by enhancing persistent Na^+ currents (I_{NaP}) from Na^+ channels. The increase in positive charge is balanced by expulsion of cytosolic K^+ content. However, ROS decrease K^+ efflux from the cytosol by inactivating voltage-dependent K^+ channels. ROS-mediated activation of the sarc-K_{ATP} and mito-K_{ATP} channels, which increases K-efflux from the cytosol or sequesteration into the mitochondria, respectively. This may compensate for some of the increased positive charge in the cytosol. Overall, increased positive charge in the cytosol may prolong the depolarization phase of the action potential and reduce the excitability.

influencing depolarization, whereas the rapid and slow delayed rectifier K+ currents (I_{Kur}, I_{Kr}, and I_{Ks}), the transient outward current (I_{Kto}), and the inward rectifier potassium current (I_{K1}) cause outward currents. Any irregularity in these currents will lead to an impaired ionic balance in the cell, alteration in action potential, and may cause EADs, DADs, and TA, that is, arrhythmia. Eventually, this may affect the cellular excitability.

From various studies, it can be summarized that ROS increase or decrease I_{CaL} with a net result of an increased intracellular Ca^{+2} *via* its action on other Ca^{+2}-handling proteins such as RyR, SERCA, and NCX. Since ROS reduce the ability of SERCA and PMCA to sequester the cytosolic Ca^{+2} back into the SR or expel out of the cytosol, respectively, the cytosolic Ca^{+2} would remain high. In this regard, it is to be considered that although ROS affect the functions of PMCA and SERCA by direct modulation, the activities of these channels depend upon the cellular ATP (149) as well. Enhanced ROS in the cytosol is accompanied by a decreased ATP synthesis due to less utilization of the cellular O_2 by the respiratory change (13, 65). Therefore, in presence of ROS, decreased ATP could cause the reduced activity of the ATPase pumps such as SERCA and PMCA. Regarding intracellular Ca^{+2}, NCX plays a major role in expulsion of Ca^{+2} out of the cytosol in exchange of Na^+. ROS induce the activity of NCX directly, and thus may cause expulsion of some of the Ca^{+2}. However, under the conditions of increased cellular ROS, I_{NaP} is enhanced (83). Enhanced I_{NaP} leads to an increased intracellular Na^+ that triggers the reverse mode of the NCX. Thus, instead of clearing Ca^{+2} out of the cell, in presence of ROS, NCX may actually increase the cytosolic Ca^{+2} in exchange of Na^+ (140). In addition, elevated cytosolic Na^+ also leads to a decreased Ca^{+2} uptake by the mitochondria, perhaps due to the impairment of the mitochondrial NCX. The interplay between cytosolic and mitochondrial NCX and increased intracellular Na^+ is complex and not well understood primarily, because their activities are modulated by the intracellular ROS differently. Overall, ROS enhance the depolarizing inward currents and thus cause increase in the intracellular Ca^{+2} and Na^+ in the cell. This may result in a decrease in the repolarizing reserve (defined as the ability of the cells to repolarize as ion channels or current density varies) and thus decrease opportunity for the cells to return to the resting potential that decreases excitability, and may lead to action potential prolongation promoting arrhythmias.

The majority of the repolarizing reserve of the cardiac cells is provided by the K^+ channels that cause an outward K^+ current. ROS reduce all the voltage-dependent K^+ currents I_{Kto}, I_{Kur}, and I_{Ks}, thereby further reducing the repolarizing reserve. However, ROS increase the outward I_{Kr} at depolarizing voltages that may contribute to normalizing repolarization reserve. The Kv11.1 channels and thus I_{Kr} are not activated until a substantial repolarizing voltage has been reached by the activity of I_{Ks}. Therefore, in a myocyte in which inward currents (I_{Na} and I_{Ca}) have increased and majority of the outward currents (in particular I_{Kto} and I_{Ks}) have decreased, the depolarizing phase of the action potential has already prolonged before even the I_{Kr} gets activated. Nevertheless, it has been shown that inhibition of all the inward K^+ currents together has a more potent effect on reducing the repolarization reserve in the myocytes than reduction of only one type of K^+ current (29). Furthermore, a decrease in membrane conductance during the plateau phase due to the inhibition of I_{Kv} would make the cell more sensitive to even small depolarizing currents such as the I_{NCX}. The net effect would be prolonged action potential duration. In this regard, previous studies analyzing the mechanisms of ROS-induced prolongation of the action potential have implicated not only the role of increased I_{NaP} and a decreased I_{CaL} that ultimately causes increased intracellular Ca^{+2} but also the role of decreased I_{Kv} (57). Indeed, the time course of I_{Kv} reduction by ROS closely paralleled the effects on the action potential duration as shown by Cerbai *et al*. (44). This is consistent with the notion that a decrease in the K^+ current responsible for membrane repolarization (at potential values around the plateau phase) may favor early after depolarization development.

Another important aspect of the cellular ROS is the induction of K_{ATP} channels. K_{ATP} channels when open, due to an increase in cellular pH or a decrease in ATP content, contribute to the cellular repolarizing currents by expulsion of K^+ from the cells (30). Cellular ROS directly open the sarc-K_{ATP} channels causing a net outward K^+ current, thereby depolarizing the membranes and prolonging the action potential. Furthermore, ROS also cause opening of the mito-K_{ATP} channels, which increases the K^+-flux into the mitochondria, causing the respiratory chain inhibition due to shifting of ionic balance and thus shuts the ATP synthesis resulting in a collapse of the $\Delta\Psi_m$ (87). Because the respiratory chain is inhibited, and ATP synthesis has stopped, the synthesis of ROS increases even further. This has been shown to occur in a network of mitochondria that are closely attached to each other in the cells, wherein the ROS from one mitochondria influence the ROS generation from the neighboring mitochondria, and this leads to the oscillations of $\Delta\Psi_m$ (13). The net effect is collapse of the $\Delta\Psi_m$, decreased cellular ATP that opens sarc-K_{ATP}, and mitochondrial ROS-induced ROS release that causes oscillations of the $\Delta\Psi_m$ (12). Decreased cellular ATP will contribute to a decreased SERCA and PMCA activity as well as opening of the sarc-K_{ATP}, both of which cause prolongation of the depolarizing phase of the action potential. Moreover, collapse of the $\Delta\Psi_m$ and oscillation of the $\Delta\Psi_m$ will further lead to arrhythmogenesis as explained by O'Rourke *et al*. (13). In summary, ROS generation in the cardiac cells alters the activity of various ion channels in a way such that the net inward currents are increased, and the net outward currents are decreased. This results in the enhanced cellular Na^+ and Ca^{2+} ions, prolongation of the action potential duration, reduction of the refractory phase, and thus reduced excitability that promotes arrhythmogenesis. Therapies targeting decrease in ROS may lead to a safer and more general cure for various arrhythmogenic diseases, as has already been indicated in several clinical studies.

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Abbreviations Used

ASK-1 = apoptosis-regulating signal kinase-1
BCNU = 1,3-bis-(2-chloroethyl)-1-nitrosourea
bEAG = bovine ether-a-gogo gene
BK_{Ca}, = large conductance K_{Ca}
BSO = buthionine sulfoximine
Ca-CaM = calcium–calmodulin
CaMKII = CaM kinase II
CAT = catalase
cGMP = cyclic guanosine monophosphate
CREB = CRE-binding protein
CTS = cardiotonic steroids
DADs = delayed afterdepolarizations
DHF = dihydroxyfumarate
DIP = diphenylene iodonium
DTDP = 2,2′-dihydropyridine
DTNB = 5,5′-dithio-bis(2-nitrobenzoic acid)
DTT = dithiothreitol
EADs = early afterdepolarizations
EGFR = epidermal growth factor receptor
ERK = extracellular signal-regulated kinase
ET-1 = endothelin-1
FKBP = FK-506-binding proteins
GPx = glutathione peroxidase
GSH = glutathione
GSSG = oxidized glutathione
HERG = human ether-a-gogo-related gene
20-HETE = 20-hydroxyeicosatetraenoic acid
HOCl = hypochlorous acid
IK_{Ca} = intermediate conductance K_{Ca}
IMAC = inner membrane anion channel
K_{Ca} = calcium-activated K
KChAP = K-channel-associated protein
KChIP = K-channel-interacting protein
KO = knockout
MAPK = mitogen-activated protein kinase
MsrA = methionine sulfoxide reductase
MTSE = methanethiosulfonate ethylammonium
NAC = N-acetylcysteine
NCX = Na^+/Ca^{+2} exchanger
NOS = nitric oxide synthase
$O_2^{\bullet-}$ = superoxide radical ion
$OH^{\bullet}$ = hydroxyl radical
PKC = protein kinase C
PMA = phorbol 12-myristate 13-acetate
PUFAs = polyunsaturated fatty acids
RNS = reactive nitrogen species
ROS = reactive oxygen species
RTK = receptor tyrosine kinases
RyR = ryanodine receptor
SERCA = sarcoendoplasmic reticulum Ca^{+2}-ATPase
SH = sulfhydryl
SIN = 3-morpholinosydnonimine-HCl
SK_{Ca} = small conductance K_{Ca}
SOD = superoxide dismutase
SR = sarcoplasmic reticulum
SUR = sulfonylurea receptor
t-BHP = tertbutyl hydroperoxide
TA = triggered activity
TTX = tetrodotoxin
XOR = xanthine oxidoreductase