Evidence for the regulation of L-type Ca^{2+} channels in the heart by reactive oxygen species – mechanism for mediating pathology

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Summary

1. It is well recognised that reactive oxygen species (ROS) can activate transduction pathways to mediate pathophysiology. An increase in ROS has been implicated in a number of cardiovascular disorders. ROS regulate cell function through redox modification of target proteins. One of these target proteins is the L-type Ca^{2+} channel.

2. There is good evidence that thiol reducing and oxidising compounds including hydrogen peroxide can influence calcium channel function. The evidence for regulation of the channel protein and regulatory proteins by thiol specific modifying agents and relevance to hypoxia and oxidative stress will be presented.

3. Clinical studies suggest that calcium channel antagonists may be beneficial in reducing myocardial injury associated with oxidative stress. The identification of cysteines as possible targets for intervention during hypoxic trigger of arrhythmia or chronic pathological remodelling is discussed.

Introduction

The production of reactive oxygen species (ROS) is usually considered to be directly detrimental to the health of organisms because ROS can damage key macromolecules such as DNA, proteins and lipids.[1] However, ROS can also act as signalling molecules able to stimulate and modulate a variety of biochemical and genetic systems including the regulation of signal transduction pathways, gene expression, proliferation and cell death by apoptosis.[2] The activity of some second messengers appears to be under the tonic regulation of ROS and this is necessary for normal function.[3,4]

Reactive oxygen species are generally speaking oxygen molecules in different states of reduction as well as compounds of oxygen with hydrogen and nitrogen. Superoxide is formed from the reduction of oxygen but since it cannot easily cross membranes its reactivity is limited to the organelle in which it is produced.[2] At physiological pH it is rapidly dismutated to hydrogen peroxide by superoxide dismutase. The biologically active reactive species are hydrogen peroxide, hydroxyl radicals, hypochlorite and peroxynitrites. Since hydrogen peroxide can readily cross plasma membranes it is considered a good candidate as a signal molecule.[5,6]

The cell normally experiences a predominantly reduced intracellular environment due to the large pool of reduced thioredoxin and glutathione.[5] An alteration in the cell's redox state such as increased ROS production is associated with pathology. The regulation of signalling pathways by hydrogen peroxide (H_2O_2) and superoxide has been linked to the development of various cardiovascular diseases. These include ischemic heart disease, hypertension, cardiomyopathies, cardiac hypertrophy and congestive heart failure[7-9]. It would appear therefore that consistent with homeostatic mechanisms necessary to maintain normal cellular function, an alteration in production of ROS may be capable of inducing pathology because the redox state of the cell or target protein has been altered.

The cellular transport of ions is vital to life. In addition to participating in a number of physiological responses, ion channels underlie the electrical activity of cells. Ion channels have a unique functional role, because not only do they participate in electrical activity, they form the means by which electrical signals are converted to responses within the cell. Calcium is a good example. Plasma membrane calcium channels such as the L-type Ca^{2+} channel activate quickly and can rapidly change the cytoplasmic environment. Once inside the cell, calcium acts as a 'second messenger' prompting responses by binding to a variety of calcium sensitive proteins. Calcium channels are known to play an important role in stimulating muscle contraction, in neurotransmitter secretion, gene regulation, activating other ion channels, and controlling the shape and duration of action potentials. Since excessive quantities can be toxic, its movement is tightly regulated and controlled. Calcium is also a mediator of pathology in that it is necessary for activation of second messengers such as MAPK, calcineurin and nuclear factor for activated T cell (NFAT) transcription signalling that are implicated in the development of cardiac hypertrophy.[10,11]

There is good evidence that ion channel function can be modified by ROS. The ion channel may be the direct target of ROS. Alternatively an intermediate regulatory protein may be the target of ROS that then modifies the function of the channel. The thiol redox state of the channel protein may be an important determinant of channel function and the cell's fate. In this article the evidence for regulation of the cardiac L-type Ca^{2+} channel by reactive oxygen species will be presented and the role for the channel as possible mediator of pathology during alterations in cellular redox state will be discussed. Specifically the evidence for altered channel function

during hypoxia and during increased production of ROS (or oxidative stress) will be presented.

The L-type Ca^{2+} channel as target of ROS

Channel structure

The L-type Ca^{2+} channel is a member of a gene superfamily of transmembrane ion channel proteins that includes voltage-gated K^+ and Na^+ channels. Ca^{2+} channels share structural similarities with K^+ and Na^+ channels in that they possess a pore-forming α_1 subunit comprising four repeats of a domain with six transmembrane-spanning segments that include the voltage sensing S4 segment and the pore-forming region (Figure 1). The α_1 subunit is large (190-250 kDa) and incorporates the majority of the known sites regulated by second messengers, toxins and drugs. This subunit is usually complexed with at least three auxiliary subunits, α_2, δ, β and γ, with the α_2 and δ subunits always linked by a disulfide bond.[12]

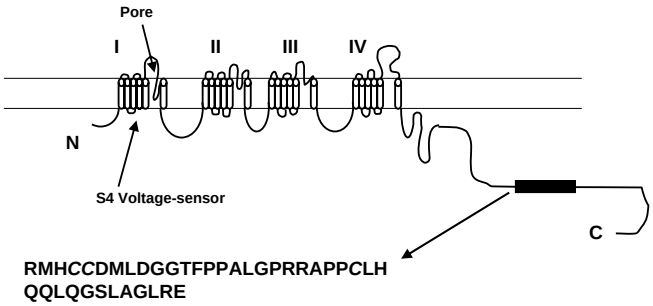

Figure 1. *The four homologous domains of the α_1 subunit of the L-type Ca^{2+} channel indicating the pore-forming region (Pore) between S5 and S6, voltage sensor S4 and region in the C terminal domain that is proposed to be oxygen sensitive with cysteines shown in Italics (see text). Predicted α helices are depicted as cylinders.*

Voltage gated calcium channels contain many cysteines. The α_1 subunit from L-type channels, for example, has 48. Cysteine residues are generally thought to be the most likely target of redox or nitrosylation modification in proteins, as free thiols can easily react with oxygen or reactive nitrogen species and can be assisted in forming intramolecular disulfide bonds[13-15]. Not all of these will be susceptible to oxidation or reactions, however, as many will already be involved in disulfide bonds.

Modulation of channel function by thiol-specific agents

In vitro studies examining the effect of thiol-specific reducing or oxidising agents on L-type Ca^{2+} channel function have demonstrated that free thiols in the protein are likely to be sensitive to the oxidation state of the cell[16-22]. In guinea pig ventricular myocytes, the thiol-reducing agent dithiothreitol decreases basal native L-type Ca^{2+} current while 5,5′-dithio-bis[2-nitrobenzoic acid] (DTNB), a thiol-specific oxidising agent increases basal current.[17] Similarly in frog ventricular myocytes, thiol oxidising agents increase L-type Ca^{2+} current.[23] The effect can be prevented by dithiothreitol but is unaffected by an inhibitor of cAMP or application of GDPβS implying that a regulatory protein is not involved in the response. In contrast the oxidising mercury compound p-hydroxy-mercuric-phenylsulphonic acid (PHMPS) has been reported to decrease basal L-type Ca^{2+} current.[20] Dithiothreitol has no effect on human cardiac L-type Ca^{2+} channels expressed in HEK 293 cells but reverses the inhibitory effects of another oxidising agent thimerosal.[19] Therefore thiol oxidising or reducing agents can affect channel function but the responses appear to vary. This may be due to a contribution by the auxiliary subunits of the native channel that is absent in the expressed (cloned) channel in which only the α subunit is present. For example the auxiliary β_3 subunit of Ca^{2+}-dependent K^+ channels participates in movement of ions through the α subunit by forming gates that block pore access.[24] This gating mechanism is abolished by reduction of extracellular disulfide linkages. Therefore auxiliary subunits may play important regulatory roles where reactive cysteines alter subunit function.

How is a reactive thiol on the channel relevant to pathology?

In cardiac myocytes rapid changes in cellular oxygen and generation of ROS can contribute to electrophysiological instability in the myocardium and the development of arrhythmias.[25-30] Understanding how ion channels respond to changes in oxygen tension may help with developing strategies to prevent the trigger of arrhythmias. Acute hypoxia (rapidly decreasing O_2 pressure to approx 15 mmHg without depletion of cellular ATP) causes a decrease in basal L-type Ca^{2+} current in cardiac myocytes.[17,18,31-33] This has also been demonstrated in the recombinant α_{1C} subunit of the L-type Ca^{2+} channel.[19,34,35] In addition the region of the α_{1C} subunit that is responsive to hypoxia has been proposed (Figure 1). Only one of three splice variants expressed in the heart (hHT variant) appears to confer sensitivity to hypoxia.[35] In the case of the native L-type Ca^{2+} channel, dithiothreitol mimics the effects of acute hypoxia (PO_2 17mmHg) and DTNB attenuates the hypoxic inhibition of basal channel activity.[17] In addition exposure of myocytes to catalase (that specifically converts hydrogen peroxide to water and oxygen) mimics the effect of hypoxia and the effect of dithiothreitol.[32]

Hypoxia and cellular ROS production

Although controversial, acute hypoxia (that does not necessarily deplete cellular ATP) has been reported to be associated with a decrease in ROS.[32,36-38] This is controversial because some studies have reported that ROS increase during hypoxia.[39-41] However the duration and degree of hypoxia appears to be an important determinant of ROS production. In the mitochondria, the reduced state of the electron transfer proteins, the mitochondrial membrane potential and the level and duration of hypoxia/anoxia are critical in determining production of

reactive oxygen species. On the other hand production of superoxide by NAD(P)H-oxidase is dependent upon oxygen as a substrate. If available oxygen decreases superoxide production also decreases. Therefore, it is important to stress that not all hypoxic conditions are alike and cells appear to respond differentially to oxygen deprivation depending on the site of ROS generation in the cell, the cells' intrinsic requirements for oxygen and the duration of hypoxia. In adult ventricular myocytes a decrease in O_2 pressure from 150 mmHg (room air) to 15 mmHg results in a 41% decrease in superoxide (assessed with the fluorescent indicator dihydroethidium)[33] and a significant decrease in cellular hydrogen peroxide (assessed with 5-(and -6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester).[32] The alteration in production of reactive oxygen species in cardiac myocytes in response to acute hypoxia would appear to be due to altered mitochondrial function and not an alteration in activity of NAD(P)H-oxidase.[32,33] Therefore there is good evidence that cellular ROS production decreases during hypoxia and that a reduction in cellular redox state that occurs with hypoxia can significantly alter channel function.

A decrease in calcium influx during the plateau phase of the action potential may be necessary to prevent arrhythmias associated with prolongation of the QT interval during hypoxia that occurs without activation of ATP-sensitive K^+ channels.[42] Therefore a decrease in L-type Ca^{2+} channel basal current may be adaptive during hypoxia. However, hypoxia also increases the sensitivity of the L-type Ca^{2+} channel to the β-adrenergic receptor agonist isoproterenol and this may not be beneficial to the cell.[17,32,33] Typically β-adrenergic receptor stimulation increases channel activity through activation of cAMP and subsequent protein kinase A-dependent phosphorylation of the channel.[43] This increases the mode 2 state of channel openings (long openings). During cardiac ischemia there is an increase in sympathetic drive and circulating catecholamines. An increase in the sensitivity of the channel to β-adrenergic receptor stimulation would be expected to cause a prolongation in action potential duration that is usually associated with life-threatening arrhythmias. This may explain the increased incidence of early afterdepolarisations (that typically precede life-threatening ventricular tachyarrhythmias) associated with hypoxia and ischemia.[17,30] The increase in sensitivity of the channel to isoproterenol can be mimicked by dithiothreitol[17] and perfusing cells intracellularly with catalase.[32] Consistent with this, exposing myocytes to the sulfhydryl oxidant phenylarsine oxide, is associated with a decrease in sensitivity of the channel to isoproterenol.[44] Therefore increased sympathetic stimulation to the heart during ischemia and hypoxia may trigger arrhythmia as a result of altered sensitivity of the L-type Ca^{2+} channel to β-adrenergic receptor stimulation. The increase in sensitivity of the channel to β-adrenergic receptor stimulation does not involve nitric oxide and occurs downstream from the β-adrenergic receptor at the level of protein kinase A or the channel protein.[17] Since the activity of protein kinase A can be altered by oxidation of thiols on the protein, protein kinase A may be an important regulator of cell function during changes in redox state.[45-47] Understanding how the channel and β-adrenergic receptor pathway are altered during hypoxia would assist in identifying a target site for designing antiarrhythmic drugs to prevent trigger of arrhythmia during ischemic events.

Oxidative stress and regulation of the channel

Hydrogen peroxide can also directly alter channel function. Oxidation of the channel by hydrogen peroxide enhances L-type calcium channel activity.[32,48] The enhanced calcium influx suggests oxidation of the channels may contribute to alterations in calcium homeostasis during oxidative stress. The generation of ROS by the mitochondria is dependent on intracellular Ca^{2+} and enhanced production of ROS occurs with enhanced mitochondrial matrix Ca^{2+} uptake.[49,50] As mentioned above the regulation of signalling pathways by ROS has been linked to the development of various cardiovascular diseases. In addition Ca^{2+} is an important mediator of cell growth and a persistent increase in intracellular Ca^{2+} is associated with disease states such as heart failure.[51] Therefore Ca^{2+} and ROS participate in pathological remodelling. However the mechanisms by which they cooperate in mediating pathology are poorly understood.

Exposing cardiac myocytes to ROS further increases mitochondrial ROS production[28,50] (Figure 2). It has been proposed that ROS production increases due to a direct effect of ROS on mitochondrial function. Alternatively ROS production may increase due to increased calcium uptake into the mitochondria as a result of enhanced calcium influx through the L-type Ca^{2+} channel. We exposed guinea pig ventricular myocytes to a transient oxidative stress (that mimics the burst of ROS that occurs *in vivo* with ischemia-reperfusion) and examined the effect on cellular ROS and calcium. Exposure to 30 μM hydrogen peroxide for 5 min followed by 10U/ml catalase for 5 min to degrade the hydrogen peroxide caused a 65% increase in dihydroethidium (DHE) signal without induction of apoptosis (assessed by caspase 3 assay) or necrosis (assessed by propidium iodide uptake) in the cells (data published as an abstract *Circ. Res.* 2006, 99:E19-E50). Exposure of the myocytes to nisoldipine (L-type Ca^{2+} channel antagonist) or Ru360 (an inhibitor of the mitochondria calcium uniporter) before or after the 5 min exposure to 30 μM hydrogen peroxide significantly attenuated the increase in DHE signal. However dantrolene, an inhibitor of sarcoplasmic reticulum calcium release did not attenuate the increase in DHE signal. This suggested that an increase in calcium influx through the channel and increased calcium uptake by the mitochondria were necessary for the increase in cellular superoxide. We tested this directly by activating the channel with the pharmacological agonist (-)-Bay K8644. An increase in influx of calcium through the channel alone was sufficient to increase cellular superoxide by 79%. In addition L-type Ca^{2+} channel basal current density was significantly increased from 5.4 to 8.9 pA/pF after transient exposure to

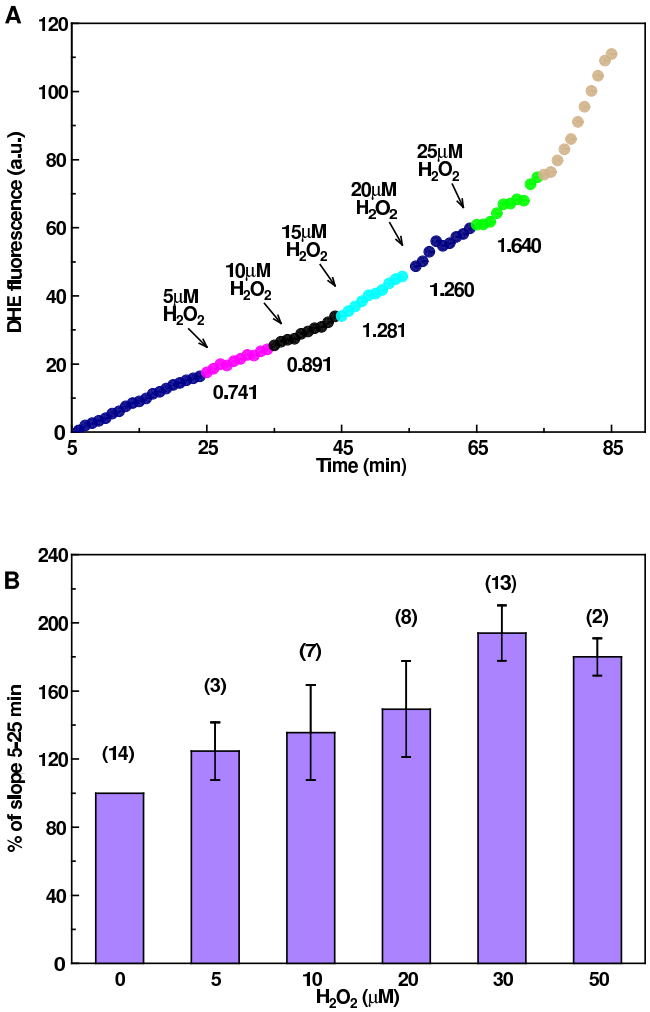

***Figure 2**. Application of hydrogen peroxide externally causes a further increase in cellular superoxide assessed with the fluorescent indicator dihydroethidium (DHE). **A:** DHE recorded from a guinea pig ventricular myocyte prior to and following exposure to increasing concentrations of H_2O_2 as indicated. Slope values are indicated below each H_2O_2 concentration. **B:** Mean±SE of ratio of fluorescence expressed as % of slope recorded at 5-25 min for guinea pig ventricular myocytes exposed to concentrations of H_2O_2 as indicated. Number of cells tested is shown in parentheses above each bar. 25-30 μM H_2O_2 consistently increased cellular superoxide without apoptosis or necrosis (for details see text).*

hydrogen peroxide. This effect persisted for up to 9 hours after the transient exposure to hydrogen peroxide. Therefore it would appear that transient oxidation of the channel by hydrogen peroxide is sufficient to increase superoxide from the mitochondria as a result of increased calcium influx through the L-type Ca^{2+} channel. The effect persists because a positive feedback exists between increased basal channel activity, elevated intracellular calcium and superoxide production by the mitochondria (Figure 3). This may be sufficient to activate hypertrophic signalling pathways and induce pathological remodelling.

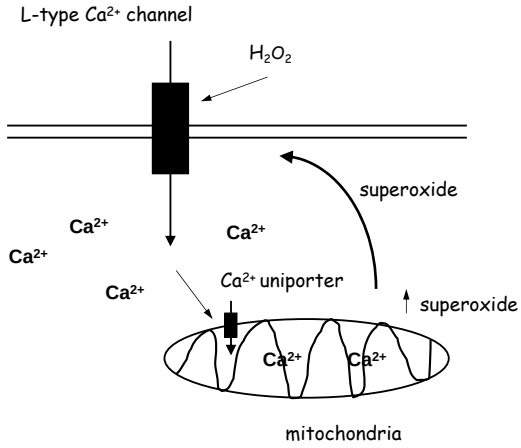

***Figure 3**. Model explaining persistent increase in cellular superoxide and increase in L-type Ca^{2+} channel basal current density as a result of a transient oxidative stress. Transient exposure to extracellular hydrogen peroxide oxidises the channel causing an increase in channel basal current density. An increase in calcium uptake into the mitochondria follows (as a result of an increase in calcium influx through the L-type Ca^{2+} channel) that further increases superoxide production by the mitochondria. The increase in cellular superoxide causes further oxidation of the channel (for further details see text).*

The channel as therapeutic target

Results from clinical studies have suggested that the channel may be an attractive target for treatment of myocardial injury during oxidative stress. A combination of calcium channel blockers and antioxidants are effective in reducing the progression of atherosclerosis that involves increased ROS production and lipid peroxidation in the vasculature.[52,53] Administration of the L-type Ca^{2+} channel antagonist verapamil before coronary perfusion with thrombolytic therapy improves recovery of the region around the infarct[54] and is associated with lower restenosis after percutaneous coronary intervention.[55] In animal studies calcium channel antagonists have been effective in reducing myocardial oxidative stress in stroke prone hypertensive rats,[56] protecting against myocardial reperfusion injury[57] and stunned myocardium.[58] Therefore the channel represents an attractive candidate for targeting under conditions of oxidative stress.

Conclusions

There is now good evidence that ROS can act as signalling molecules to mediate pathology. ROS can also regulate L-type Ca^{2+} channel function most likely as a result of direct redox modification of cysteines on the channel protein, although redox modification of regulatory proteins may also influence channel function. Cellular responses to changes in oxygen tension such as hypoxia are

generally thought to be adaptive to prevent the deleterious effects of systemic hypoxemia. However acute changes in cellular ROS production and redox state can also represent a trigger for arrhythmia as a result of acute alterations in channel function. Pathological remodelling may involve persistent oxidation of the channel, increased ROS and increased calcium influx during oxidative stress. In order to treat disorders that involve modulation of channel function during alterations in redox state a greater understanding of how the channel protein is affected is required. Greater knowledge of the structural details of calcium channel regulation by ROS is an important step in designing drugs that could target specific sites on the protein. If a cysteine or cysteines are altered and identified this would provide a potential site to target for modification of channel function.

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