HYPOXIC REGULATION OF RECOMBINANT L-TYPE Ca^{2+} CHANNELS

AN EXAMINATION OF THE MECHANISMS UNDERLYING ACUTE AND CHRONIC HYPOXIC REGULATION OF L-TYPE Ca^{2+} CHANNEL α_{1C} SUBUNITS

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ABSTRACT

L-type Ca^{2+} channels, found in vascular smooth muscle cells, function to control Ca^{2+} influx, which directly regulates the degree of tension in the vasculature. An influx of Ca^{2+} causes these cells to contract while inhibition of this channel causes muscle relaxation, a major goal in treating hypertension. Acute hypoxia inhibits, and chronic hypoxia enhances, Ca^{2+} channel currents. The mechanisms underlying these hypoxic responses were examined in HEK 293 cells by altering cellular levels of proposed mediators of O_2 sensing which have previously been shown to be involved in the redox model of O_2 sensing in various cell types. In these studies I investigated the roles of mitochondrial complexes and NADPH oxidase function, and changes in cellular ROS levels, on the acute and chronically hypoxic regulation of recombinant L-type Ca^{2+} channels. An increase in H_2O_2, a form of ROS, by exogenous application was found to enhance Ca^{2+} currents. However neither catalase nor H_2O_2 affected the acute hypoxic response. In contrast superoxide dismutase (SOD) abolished hypoxic inhibition of recombinant L-type Ca^{2+} channels, suggestive of a role of O_2- production in O_2 sensing. Altered production of this ROS during hypoxia may occur within the mitochondria since acute O_2 sensing was abolished in mitochondria-depleted ρ^0 cells. Alterations in NADPH oxidase activity via application of NADPH oxidase inhibitors such as DPI and PAO did not mediate the acute hypoxic response. Hypoxic regulation of mitochondrial complex I may also mediate the response to chronic hypoxia since current enhancement by this stimulus was abolished by rotenone. These findings support the involvement of

altered mitochondrial function in the O_2 sensing pathway which mediates the hypoxic responses of recombinant L-type Ca^{2+} channel α_{1C} subunits.

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LIST OF ABBREVIATIONS

O_2 - oxygen

H_2O_2 – hydrogen peroxide

O_2^- - superoxide anion

SOD – superoxide dismutase

ROS – reactive oxygen species

ETC – electron transport chain

DPI – diphenylene iodonium

PAO – phenylarsine oxide

CH – chronic hypoxia

NADPH – nicotinamide adenosine dinucleotide phosphate

NADH - nicotinamide adenine dinucleotide

GSSG – oxidized glutathione

GSH – reduced glutathione

Ca^{2+} - calcium ion

K^+ - potassium ion

Na^+ - sodium ion

NEB – neuroepithelial body cells

PC12 – pheochromocytoma cells

HEK – human embryonic kidney

ATP – adenosine triphosphate

GENERAL INTRODUCTION

O_2 is an absolute necessity for higher level organisms to carry out fundamental physiological processes, the most important of which is the production of energy via mitochondrial-generated ATP. Even slight decreases in O_2 levels for short amounts of time can have serious negative consequences for an organism, and can lead to cell death if the organism is unable to elicit the appropriate response to counteract the hypoxic stimulus. Changing activity levels of an organism are also directly related to the amount of oxygen required for tissue and organ utilization. An organism must possess mechanisms by which they can sense O_2 levels to ensure that enough O_2 is circulating in their blood to match physiological demand. If insufficient O_2 is being supplied, corrective action must be taken to restore O_2 supply. The ways in which an organism can restore O_2 supply include increasing breathing rate/depth, vasodilatation of peripheral arteries or constriction of pulmonary arteries (Boron and Boulpaep, 2003). These actions are aided in part by activating or modifying ion channels. Ion channels located in chemosensory cells respond to low O_2 levels and act as an integral part of signaling mechanisms that work to compensate for hypoxia. For example, K^+ channels in the carotid body become inhibited during hypoxia, which depolarizes the cell membrane, leading to an increase in Ca^{2+} influx through voltage-gated Ca^{2+} channels and frequently to the release of neurotransmitters that signal the brain to activate mechanisms that improve ventilation (Gonzalez *et al.*, 1994; Peers, 1997). Although it is known that ion channels can be modulated by hypoxia, the exact mechanism underlying hypoxic

regulation of ion channels remains to be understood. This thesis focuses on a specific ion channel, the L-type Ca^{2+} channel, and investigates how this channel responds to hypoxia as well as the factors that modulate this response. In order to understand this, a detailed literature review pertaining to this thesis is provided below.

HEK 293 cells

HEK 293 cells are derived from human embryonic kidney cells that have been transformed by sheared adenovirus type 5 DNA (Graham *et al.*, 1977). These cells are widely used in transfection experiments and expression systems that study various aspects of cell biology. HEK 293 cells have recently been found to express a wide array of neuronal markers (Shaw *et al.*, 2002) that closely resemble neuronal derived cells prior to experimentation and have been found to express gene transcripts for a variety of ion channels including inward-rectifier K^+ channels, delayed rectifier K^+ channels and Ca^{2+} activated K^+ channels (Shaw *et al.*, 2002). DNA microarray analysis has revealed that they do not normally express the major pore-forming subunits of voltage-gated Ca^{2+} channels but do express mRNA for associated subunits of voltage-gated Ca^{2+} channels such as β-subunits and γ subunits (Shaw *et al.*, 2002). In addition, HEK 293 cells were found to be remarkably similar in phenotype and genotype to PC12 cells (Shaw *et al.*, 2002). PC12 cells are generated from pheochromocytoma cells of the rat adrenal medulla, which undergo terminal differentiation upon nerve growth factor treatment and have been used to study cellular responses to hypoxia (Taylor and Peers, 1998; Del Toro

et al., 2003; Yuan *et al.*, 2004). The use of this expression system is a powerful tool for studying ion channels, since it is possible to use this system to express a variety of ion channels and subunits including K^+, L-type Ca^{2+}, T-type Ca^{2+}, Cl^- channels among others (Cribbs *et al.*, 1998; Petersen and Nerbonne, 1999; Fearon *et al.*, 1999; Rosenbohm *et al.*, 1999). In addition, expression systems using HEK 293 cells aid in elucidating ion channel mechanisms of action, regulatory factors and other biophysical properties.

L-type Ca^{2+} channels

L-type Ca^{2+} channels are found primarily in cardiac muscle tissue and vascular smooth muscle (Fearon *et al.*, 1997; Gollasch and Nelson, 1997; Fan *et al.*, 2000; Larsen *et al.*, 2002; Boron and Boulpaep, 2003). They are a subtype of voltage-gated Ca^{2+} channel that become activated at higher voltages and produce a longer lasting depolarization compared to other types of voltage-gated Ca^{2+} channels, such as T-type Ca^{2+} channels (Hille, 2001). L-type Ca^{2+} channels are composed of numerous subunits, the primary of which is the pore-forming α_1 subunit. This subunit is composed of 4 repeating domains, termed I to IV, of 6 alpha-helical transmembrane segments termed S1 to S6. The positively-charged S4 region is thought to contain the voltage sensor which senses the electrical difference across the membrane and modifies channel gating accordingly. The S5/S6 regions form the pore of the channel, which acts as the ion gateway, allowing Ca^{2+} ions to enter the cell upon membrane depolarization (Boron and Boulpaep, 2003; Cibulsky and Sather, 2003). Five subunits make up the entire L-type

Ca^{2+} channel; α_1, the major pore-forming subunit and the auxiliary subunits; α_2, β, δ and γ , the latter of which is only found in skeletal muscle tissue (Boron and Boulpaep, 2003). The auxiliary subunits work to modulate the properties of the channel complex (Streissnig, 1999; Wei *et al.*, 2000). Upon membrane depolarization, the L-type Ca^{2+} channel becomes activated around -40 mV. Activation of the channel changes its conformation such that Ca^{2+} ions are permitted to enter the cell. The inward flux of Ca^{2+} ions depolarizes the inner side of the cell, which then causes the channel to inactivate in a slow manner. Channel inactivation changes the channel conformation back to the original closed state and Ca^{2+} ions are no longer permitted to enter the cell.

Physiological importance of L-type Ca^{2+} channels

The function of the L-type Ca^{2+} channel is to permit Ca^{2+} entry into excitable cells in a controlled and timely manner. These channels play an important role in excitation/contraction coupling, gene regulation and calcium homeostasis (Wolf *et al.*, 2003). In vascular smooth muscle cells, these channels activate the contractile mechanism such that when Ca^{2+} enters the cell, vascular smooth muscle cells contract. The same is true in cardiac muscle cells that rely on Ca^{2+} entry into myocytes for propagation of the cardiac action potential and also contraction. This action potential is necessary for cardiac contractions to take place and therefore for circulation of the blood to occur (Head and Gardiner, 2003). Dysregulation of Ca^{2+} homeostasis, either by increasing or decreasing channel gating activity can lead to the development of serious

disease. For example, increased activity of Ca^{2+} channels in vascular smooth muscle can lead to the development of chronic hypertension as increased Ca^{2+} levels inside vascular myocytes cause contraction of the vasculature, which increases blood pressure (Boron and Boulpaep, 2003). In contrast, inhibition of channel gating leads to reduced Ca^{2+} entry, which in vascular smooth muscle cells, causes vasodilatation. Relaxation of the systemic vasculature is a major goal in treating people with chronic hypertension and cardiovascular disease. It is therefore, extremely beneficial to study how these channels are regulated and the mechanisms underlying this regulation.

O_2 sensing by ion channels

Investigation of ion channel responses to hypoxia is relatively new in the field of electrophysiology. The first report indicating that O_2 levels could modulate ion channel function came from a group studying carotid body type I cells (Lopez-Barneo *et al.*, 1988). In these cells, hypoxia caused reversible inhibition of K^+ currents, and this demonstration was pivotal to our understanding of how information concerning O_2 levels in the circulating blood could be relayed to the brain so as to increase breathing rate and thus, increase blood oxygenation. Research that followed found that hypoxia caused membrane depolarization via inhibition of K^+ channel current, leading to an increase in Ca^{2+} influx via voltage-gated Ca^{2+} channels and release of neurotransmitters from carotid body type I cells (Peers, 1990). Carotid bodies are composed of clusters of sensory cells (type I or glomus) that are innervated by afferent nerve fibers (Lopez-Barneo *et al.*,

2004). Neurotransmitter release relays a signal via the afferent nerves to the respiratory center in the brain that results in an increase in breathing rate (Lopez-Barneo *et al.*, 2004). Further investigation has revealed the existence of several types of ion channels in numerous types of tissues that can be modified by hypoxia. A few examples of O_2 sensitive ion channels and tissues include: voltage-gated K^+ channels in human ductus arteriousus (Michelakis *et al.*, 2002a), L-type Ca^{2+} channels in rat vascular smooth muscle (Herrera and Walker, 1998) and Na^+ channels in rat hipppocampal tissue just to name a few (Hammarstrom and Gage, 2000).

The overall unifying response of ion channels to hypoxia is through inhibition of channel gating. In most cases, hypoxia inhibits channel activity such that there is a decrease in channel current amplitudes. For example, K^+ channels in carotid body glomus cells reduce the amount of K^+ ions effluxed from the cell (Buckler and Vaughan-Jones, 1994; Wyatt and Peers, 1995) while in systemic arterial myocytes, Ca^{2+} channels respond by reducing the amount of inward Ca^{2+} current (Franco-Obregon *et al.*, 1995; Franco-Obregon and Lopez-Barneo, 1996b). These responses are solely due to decreased Po_2 as inhibitory responses occur without modification of other variables like pH, $[Ca^{2+}]$ or ATP levels (Lopez-Barneo *et al.*, 2001). Although it has been clearly demonstrated that hypoxia modifies channel currents, the exact mechanisms of how this is accomplished remains to be understood.

O_2 sensing by L-type Ca^{2+} channels

The first studies of O_2 sensing by any other channel other than K^+ channels came from work carried out by Lopez-Barneo and colleagues, who showed that L-type Ca^{2+} channels in smooth muscle cells from various parts of the vascular system could be reversibly inhibited by acute hypoxia (Franco-Obregon *et al.,* 1995). The same group later showed that Ca^{2+} channels in proximal rabbit smooth muscle cells responded to acute hypoxia by inhibiting Ca^{2+} influx into the cell (Franco-Obregon and Lopez-Barneo, 1996a). Hypoxia has also been shown to act directly on vascular smooth muscle cells of rats to cause relaxation by inhibiting L-type Ca^{2+} channels (Herrera and Walker, 1998) in addition to inhibiting L-type Ca^{2+} channels in human coronary myocytes (Smani *et al.*, 2002). Similarly, electrical and mechanical responses in arteriole smooth muscle cells from hamster cheek pouches were directly affected by changes in Po_2 levels (Welsh *et al.*, 1998), effects also found to be mediated by L-type Ca^{2+} channels.

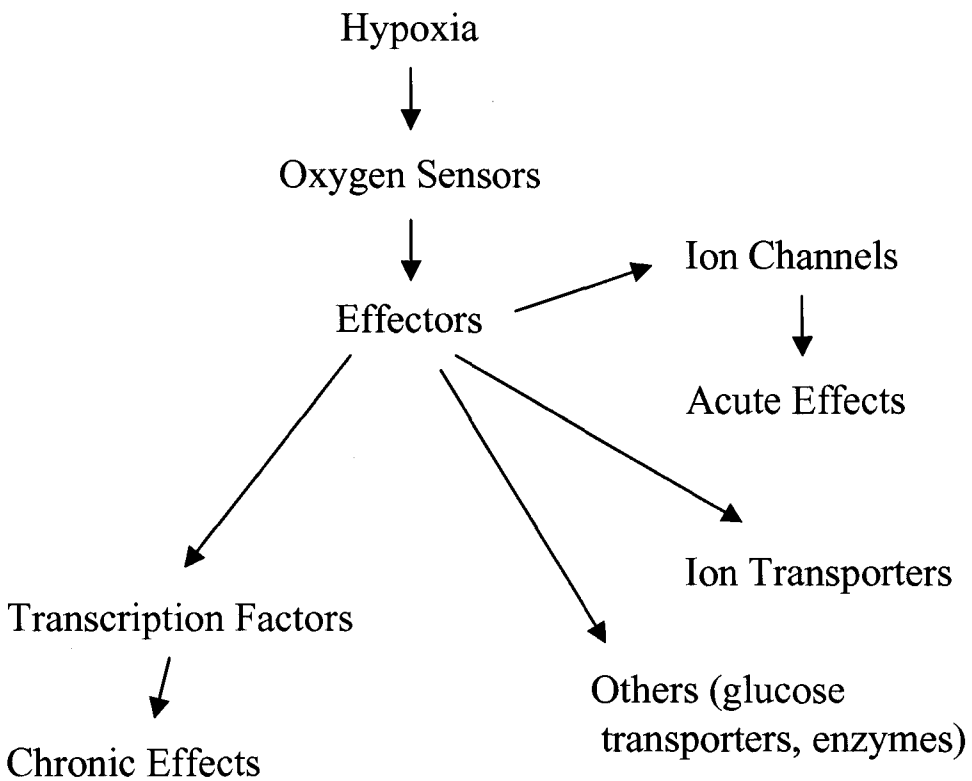
Difficulty in isolating vascular smooth muscle and cardiac cells from various animals has lead to the use of recombinant systems in which cDNA of various pore-forming channel subunits are functionally expressed in continuously propagated cell lines. It was brilliantly shown that the α_{1C} subunit of L-type Ca^{2+} channels, originally cloned from human cardiac tissue, can alone mediate the hypoxic response when stably expressed in HEK 293 cells (Fearon *et al.*, 1997). This hypoxic response was highly voltage-dependent and was indistinguishable from currents observed in native channels expressed in vascular smooth muscle (Fearon *et al.*, 1997). This was the first report to

demonstrate the use of recombinant systems in delineating mechanisms of O_2 sensing and further demonstrated that auxiliary channel subunits are not required for O_2 sensing. Fearon *et al.* (1999) later showed that cysteine residues in the α_{1C} subunit of the recombinant human cardiac L-type Ca^{2+} channel were susceptible to modulation by oxidizing and reducing agents such that channel function was altered. Further investigation into hypoxic regulation of the α_{1C} subunit came with studies that investigated splice variants of this subunit. Using three variant isoforms of the α_{1C} subunit, it was shown that a specific 39 amino acid region in the C-terminal tail of this channel was essential for O_2 sensing (Fearon *et al.*, 2000). The exact mechanism of how hypoxia is able to inhibit L-type Ca^{2+} channels has not yet been elucidated but it has also been determined that the inhibition in Ca^{2+} current amplitude by acute hypoxia is due slowing of activation kinetics without channel inactivation or deactivation being altered (Fearon *et al.*, 1997).

Proposed models of O_2 sensing

Exactly how the L-type Ca^{2+} channel senses a decrease in oxygen tension is not fully understood. The overall general theory of how acute hypoxia is sensed by cells is that sudden decreases in Po_2 levels are somehow sensed by O_2 sensors which then signal effector complexes that modify ion channel gating activity (see Figure 1). Good candidates for O_2 sensors and effector complexes must closely interact with O_2 or be directly affected by changing O_2 levels. Models for O_2 sensing propose heme-containing

Figure 1: Proposed components of the hypoxia signaling pathway. Hypoxia is detected by oxygen sensors which then signal effector molecules. Effector molecules then modify ion channel activity which produces acute effects of hypoxia, such as vasodilatation of systemic parts of the vasculature. If the hypoxic period is prolonged such as during chronic hypoxia, effector molecules modify transcription factors, ion transporters and other factors which then manifest as chronic effects of hypoxia such as increased mRNA levels of some O_2 sensitive ion channels.


Hypoxia
Oxygen Sensors
Effectors
Ion Channels
Acute Effects
Ion Transporters
Transcription Factors
Chronic Effects
Others (glucose transporters, enzymes)

proteins, NADPH oxidase and mitochondria as O_2 sensors as all are directly affected by oxygen levels. These complexes interact closely with O_2 and thus it is plausible that changes in O_2 levels could directly affect these complexes such that they modify channel gating and activity. Both NADPH oxidase and mitochondria generate reactive O_2 species (ROS), which can potentially alter the redox status of ion channels such that ion channel function is altered (see Figure 2) (Lopez-Barneo *et al.*, 2004). Proposed mediators in O_2 sensing include different forms of ROS such as superoxide anions (O_2^-) or hydrogen peroxide (H_2O_2). These compounds make excellent candidates for second messengers in O_2 sensing since they are freely diffusible compounds that react in appropriate time to account for the almost instantaneous changes in ion channel activity seen with acute hypoxia. They also interact directly with or are a product of proposed O_2 sensing complexes. Models and evidence for O_2 sensing that involve NADPH oxidase, mitochondria and ROS are described below.

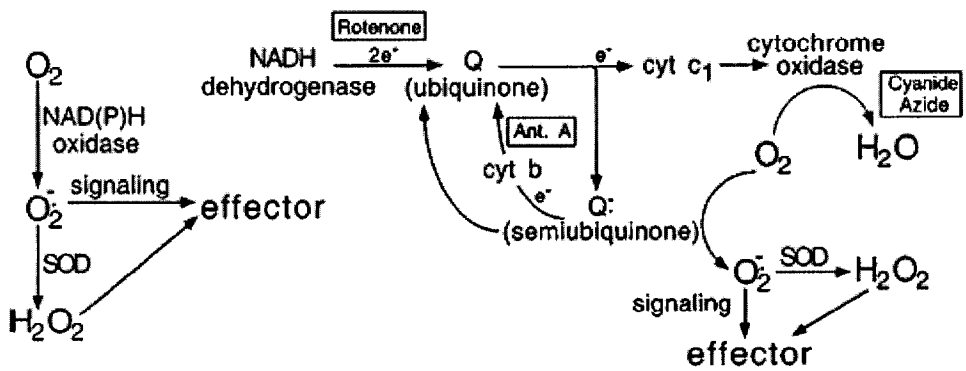
NADPH oxidase model of O_2 sensing

NADPH oxidase is a multi-subunit complex located in the cell membrane, that generates ROS by donating an electron from NADPH to O_2 molecules and results in the formation of superoxide anions (O_2^-, a reactive O_2 species) which is then dismutated to H_2O_2 by superoxide dismutase and is eventually further broken down into H_2O and O_2 (Chandel and Schumaker, 2000; Jones *et al.*, 2000; Lopez-Barneo *et al.*, 2004). H_2O_2 is known to oxidize transcription factors and other regulatory proteins such that a decrease

Figure 2: Proposed redox models of O_2 sensing. This model proposes that reactive O_2 species produced by NADPH oxidase (*left*) or complexes in the mitochondrial electron transport chain (ETC) (*right*) mediate the effects of hypoxia. NADPH oxidase donates an electron to O_2 making it O_2^-, which may either signal an effector molecule itself or become dismutated to H_2O_2 by superoxide dismutase (SOD), which then signals an effector molecule. Complexes in the mitochondrial ETC such as NADH dehydrogenase (inhibited by rotenone), semiubiquinone (inhibited by antimycin A), or cytochrome oxidase (inhibited by cyanide azide), generate O_2^- by donating electrons to O_2. O_2^- either signals an effector molecule itself or is dismutated to H_2O_2 by SOD, which then signals an effector molecule.

Redox models

Reactive oxygen species are mediators of O_2 sensing



Adapted from Lopez-Barneo, J., Pardal, R. and Ortega-Saenz, P (2001)

in O_2 levels would lead to a reduced cellular state as there is less H_2O_2 to oxidize components of the cell (Lopez-Barneo *et al.*, 2004). The NADPH oxidase model postulates that decreases in O_2 levels lead to decreased production of ROS and thus a decrease in H_2O_2 levels, shifting the redox state of the cell to a more reduced state. Exogenous reducing compounds have been shown to mimic the effect of hypoxia on O_2 sensitive K^+ (Benot *et al.*, 1993; Yuan *et al.*, 1994; Lopez-Barneo *et al.*, 2001) and Ca^{2+} currents (Fearon *et al.*, 1999). Evidence supporting the role of NADPH oxidase as an O_2 sensor comes from studies examining O_2 sensing neuroepithelial body (NEB) cells obtained from lung slice preparations in rabbits. This study showed that K^+ currents in these cells were inhibited in a similar manner to hypoxia by diphenylene iodonium (DPI), an NADPH oxidase inhibitor (Fu *et al.*, 1999). In addition, when these cells were subjected to acute hypoxia in the presence of DPI there was no further inhibition of K^+ currents suggesting that NADPH oxidase acted as an O_2 sensor in NEB's. DPI was also shown to significantly inhibit pulmonary vasoconstriction caused by exposure to acute hypoxia in isolated rat pulmonary arterial myocytes (Thompson *et al.,* 1998). NADPH oxidase was further shown to be an O_2 sensor in pulmonary neuroepithelial body cells of mice in which the NADPH subunit $gp91^{phox}$ had been genetically knocked out. Hypoxic inhibition of K^+ channels in these cells was abolished (Fu *et al.,* 2000). It was also shown that in cultured neuroepithelial cell body-derived human cell line (H146 cells) that NADPH oxidase was involved in O_2 sensing (O'Kelly *et al.*, 2000). These studies seemed promising initially by indicating NADPH oxidase as the O_2 sensor. However other studies using the same knockout model as Fu's group have shown that hypoxic

inhibition of K^+ channels was still present in mouse pulmonary artery and carotid body type I cells of mice (Archer *et al.*, 1999; He *et al.*, 2002). It was also later shown that NADPH oxidase is not the only complex that is involved in O_2 sensing in H146 cells since the NADPH inhibitors DPI and phenylarsine oxide (PAO) were found to modulate K^+ current activity but did not fully inhibit the hypoxic response of K^+ channels in these cells (O'Kelly *et al.*, 2001). Prior to research conducted in this thesis, there had been no work undertaken to investigate the role of NADPH oxidase in O_2 sensing in cells expressing recombinant L-type Ca^{2+} channel α_{1C} subunits.

Mitochondrial Theory of O_2 sensing

Mitochondria have also been proposed to play a role in O_2 sensing. Mitochondria are excellent O_2 sensing candidates because they consume most of the O_2 in cells and are a major source of O_2^- ions due to inefficiency in the electron transport chain (Lopez-Barneo *et al.*, 2001). This view was first based on the fact that cyanide, a mitochondrial complex IV inhibitor, stimulated carotid body cells to release neurotransmitters in the same fashion as hypoxia induced inhibition of K^+ current in these cells which causes neurotransmitter release (Biscoe and Duchen, 1989). In addition to this, inhibitors of the mitochondrial electron transport chain (ETC) complexes or mitochondrial inhibition was demonstrated to increase the afferent activity of the sinus nerve in carotid body glomus cells, a response observed during hypoxia (Mills and Jobsis, 1972; Mulligan *et al.*, 1981).

Later studies on carotid body cells showed decreased levels of O_2 reduced the activity of cytochrome *c* oxidase in complex IV, which caused mitochondrial depolarization, Ca^{2+} release and increased sensitivity of these cells (Streller *et al.*, 2002; Lopez-Barneo *et al.,* 2004). It was later shown that cellular responses to low O_2 levels require membrane depolarization and Ca^{2+} entry through plasmalemmal voltage-gated channels and not simply reduced activity of mitochondrial complexes (Lopez-Barneo *et al.*, 2004).

The involvement of the need for functional mitochondria in O_2 sensing has been well documented. Generation of ρ^0 cells, which lack functional mitochondrial DNA following long-term ethidium bromide treatment, from wild-type pulmonary arterial myocytes found that these cells failed to respond to hypoxia such that hypoxia-induced contraction observed in wild-type cells was attenuated in ρ^0 cells (Waypa *et al.*, 2001). Another group found that functional mitochondria are required for the transcriptional response to hypoxia (characterized by HIF-1 DNA binding, a protein whose levels increase during prolonged periods of hypoxia) (Chandel *et al.*, 1998).

Further investigation into the involvement of mitochondria in O_2 sensing has focused on redox models that involve altered function of complexes in the mitochondrial ETC. It is known that mitochondria respond to changes in Po_2 by altering production of ROS which changes the cellular redox potential and alters the redox potential of many redox-sensitive second messengers (Archer *et al.*, 1993). Archer's group demonstrated

that hypoxia caused an increase in pulmonary arterial (PA) pressure and caused simultaneous decrease in ROS as detected by chemiluminescence. Using perfused rat lungs, this group later showed that rotenone and antimycin A, inhibitors of mitochondrial complex I and III respectively, also caused a decrease in ROS and an increase in pulmonary arterial pressure, effectively mimicking the hypoxic response seen in the pulmonary vasculature. To support Archer's findings, another group later demonstrated that rotenone decreased ROS levels similar to levels observed during hypoxia (Reeve *et al.*, 2001b). Michelakis *et al.* (2002a) have recently shown that rotenone, a complex I inhibitor, mimicked the hypoxic response of K^+ channels in human ductus arteriosus smooth muscle cells. They proposed that the proximal electron transport chain is part of the O_2 sensor, at least in the cells that they were studying. Similarly, rotenone inhibited the hypoxic response of intact glomus cells of the rat carotid body but other mitochondrial complex I inhibitors did not (Ortega-Saenz *et al.*, 2003). However, this study also showed that this response was independent of electron flow and was thought to involve a rotenone-sensitive compound that acted as the O_2 sensor. The latest study has reported that rotenone mimics the effects of acute hypoxia on rat carotid body type I cells such that membrane conductance was inhibited and O_2 sensitive current response to hypoxia were abolished (Wyatt and Buckler, 2004). These findings are consistent with a mitochondrial model in which, complex I functions as an O_2 sensor by maintaining basal levels of O_2^- and H_2O_2 during normoxia and a change in Po_2 levels or mitochondrial function alters the redox state of the cell thereby altering channel function (Waypa *et al.*, 2001). There have been other studies published to date that refute the possibility that

mitochondria play a role in O_2 sensing. One such study by Searle *et al.* (2002) showed that O_2 sensing by model airway chemoreceptors (H146 cells) does not require functional mitochondria. H146 cells that lacked functional mitochondria responded to acute hypoxia in the same manner as wild-type H146 cells. To date there have been no published studies that demonstrate the role of functional mitochondria in O_2 sensing of recombinant L-type Ca^{2+} channel α_{1C} subunits.

Reactive O_2 species, ion channels and O_2 sensing

Reactive O_2 species (ROS) are defined as atoms or molecules that have one or more unpaired electrons (Gonzalez *et al.*, 2002). They are generated in the cell by NAD(P)H oxidase, mitochondrial ETC complexes and enzymes in the lysosome/phagosome and are generally present in all cell systems. Although potentially harmful to cells due to their highly reactive and unstable nature, they are used in cellular defense mechanisms to destroy bacteria and other unwanted organisms that a pose potential threat. Over-accumulation of ROS in cell can alter the redox state by oxidizing/reducing cellular components and thereby alter protein function. ROS produced by mitochondria and NADPH oxidase are proposed mediators of O_2 sensing due to the fact that they are directly affected by changing O_2 levels. ROS could act as second messengers that link the O_2 sensor to its targets (in this case L-type Ca^{2+} channels) (Chandel and Schumaker, 2000). It is also a proposed O_2 sensor as it is widely known to affect ion channel activity and function. One form of ROS, H_2O_2, has been demonstrated

to increase K^+ channel currents in human umbilical cord endothelial cells, rat brain, neuroepithelial body cells, and guinea pig ventricular myocytes (Wang *et al.*, 1996; Tokube *et al.*, 1998; Bychkov *et al.*, 1999; Shin *et al.*, 2000). Other reports have demonstrated that H_2O_2 inhibits K^+ channel opening in ductus arteriosus smooth muscle cells, renal artery endothelium and recombinant HEK 293 cells (Brakemeier *et al.*, 2003; Archer *et al.*, 2004; Tang *et al.*, 2004). L-type Ca^{2+} channel currents have also been shown to be enhanced in response to increased H_2O_2 levels (Thomas *et al.*, 1998; Akaishi *et al.*, 2004). H_2O_2 has also been demonstrated to act as a second messenger by diffusing into the cytoplasm and activating K^+ channels causing vasodilatation to occur (Porwol *et al.*, 2001). It also acts as a second messenger in cells by signaling oxidative stress (destruction of the cell by free radicals) and in addition, can also modify proteins, lipids, and DNA (Milton, 2004). It is because of this that ROS has been proposed to modulate L-type Ca^{2+} channels by changing the redox status of channel residues to alter channel function in response to acute hypoxia.

Chronic hypoxia

Prolonged periods of inadequate O_2 supply can have disastrous consequences for an organism. Humans are not normally exposed to low levels of O_2 unless they are in extremely high altitude conditions. However, there are instances in which organs and tissues may be exposed to chronically hypoxic conditions such as after a stroke or as a result of chronic obstructive pulmonary disease. Recent research into the role of chronic

hypoxia (CH) on the development of pathological disease has provided evidence linking the two. For example, repetitive instances of hypoxia are thought to underlie the development of Alzheimer's disease (Moroney *et al.*, 1996). A hallmark of Alzheimer's disease is the formation of plaques in neuronal tissue, which are composed of amyloid β peptides (Green and Peers, 2001). The amount of peptide contained within the plaques has been found to correlate with the degree of cognitive impairment (Canevari *et al.*, 2004). Amyloid β peptides are toxic to cellular function in large amounts and are characterized by an increase in oxidative stress in part due to mitochondrial dysfunction (Canevari *et al.*, 2004). CH is also known to contribute to the development of pulmonary hypertension and altered chemosensation in carotid body cells (Peers, 2002). It is proposed that CH affects the expression of ion channels, which leads to vascular remodeling and decreased sensitivity to subsequent hypoxic episodes (Peers, 2002). For example, CH has been shown to down-regulate certain K^+ channels in pulmonary smooth muscle cells leading to their depolarization and increased incidence of contraction which is a major contributing factor in pulmonary hypertension (Smirnov *et al.*, 1994). In addition to this, CH has also been shown to alter Ca^{2+} homeostasis of cells, which can lead to the proliferation of smooth muscle in the vasculature and development of hypertension (Bonnet *et al.*, 2003). The exact mechanism of how CH alters ion channel activity is not fully understood and much research is being conducted to elucidate contributing factors.

Chronic hypoxia and ion channels

CH affects ion channels in a manner unlike acute hypoxia. Acute hypoxia has an almost instantaneous affect on ion channel activity and CH primarily modifies ion channel activities through alteration in ion channel gene expression. Early studies demonstrated that Na^+ channel currents of dissociated carotid body type I cells were significantly enhanced compared to normoxic controls when cultured under chronically hypoxic conditions (Stea *et al.*, 1995). Rat pulmonary smooth muscle cells were also found to be more depolarized under chronically hypoxic conditions and this increase in depolarization was due to a selective down-regulation of K^+ channels (Smirnov *et al.*, 1994). Similarly, maxi-K^+ channel expression was found to be suppressed under chronic hypoxia in rat carotid type I cells (Wyatt and Peers, 1995). Subsequent studies have shown that prolonged periods of hypoxia significantly reduced mRNA levels of voltage-gated K^+ channel a subunits which was thought to contribute to the observed decrease in outward K^+ currents in pulmonary arterial smooth muscle cells (Wang *et al.*, 1997), a result confirmed by another group who demonstrated that pulmonary arterial myocytes subjected to CH were found to have decreased voltage-gated K^+ channel expression and function (Platoshyn *et al.*, 2001). In addition to affecting K^+ channels, CH has also been shown to affect Na^+ and Ca^{2+} channels. CH increased the current density through Na^+ channels in carotid body type I cells (Hempleman, 1996).

Early studies on the effects of CH on Ca^{2+} channels demonstrated that Ca^{2+} current was enhanced under CH conditions compared to normoxic conditions in type I

carotid body cells (Peers *et al.*, 1996). This enhancement of currents was thought to be due to the increased capacitance of the cell as current densities were similar in both cell types. This study also demonstrated that L-type Ca^{2+} channels were partly responsible for the enhancement of current as application of nifedipine, an L-type Ca^{2+} channel blocker, negated current enhancement. Current recordings in PC12 cells exposed to CH for 24 hrs prior to physiological recording, exhibited increased current densities through L-type Ca^{2+} channels which was thought to be mediated by an increase in amyloid β peptide levels (Green and Peers, 2001). Levels of amyloid β peptide has been pathologically linked to increases in ROS and further studies have examined the involvement of ROS on Ca^{2+} currents in response to CH. Green, Boyle and Peers (2002) demonstrated that chronic hypoxia can selectively up-regulate native Ca^{2+} channels in PC12 cells via increased ROS production. In addition, CH has been shown to up-regulate T-type Ca^{2+} channels and increase currents in response to hypoxia (Del Toro *et al.*, 2003). Furthermore, NADPH oxidase and/or mitochondrial derived ROS have been thought to play a role in O_2 sensing and gene regulation in CH. Evidence for this has come from studies in which inhibitors of NADPH oxidase or complexes in the mitochondrial ETC have decreased the expression of genes regulated by chronic hypoxia (Gleadle *et al.*, 1995; Ehbelen *et al.*, 1998). In addition, exogenous application of H_2O_2 was shown to inhibit production of chronic hypoxia-induced erythropoietin production (Fandrey *et al.*, 1994)

Thus, it has been demonstrated that CH affects numerous types of ion channels, either by affecting channel activity or affecting levels of ion channel gene expression.

The exact mechanism of how CH might regulate gene expression is still being investigated and factors that may be involved are still being elucidated.

Goals of this thesis

The primary goal of this thesis was to identify factors/agents that regulate the activity of L-type Ca^{2+} channel α_{1C} subunits. In addition to this, factors elucidated to regulate channel activity were also tested for their effect on channel activity during either acute or chronic hypoxia. Experiments performed to identify these factors were carried out on HEK 293 cells stably expressing the recombinant cardiac hHt isoform of the L-type Ca^{2+} channel α_{1C} subunit. Various models of the mechanisms and factors that contributed to O_2 sensing have been proposed, most notably models that include NADPH oxidase, mitochondria and/or the ROS that these complexes generate. There has been evidence that both support and refute the involvement of these complexes, the underlying reasons for the discrepancies stemming from the fact that different cell types/tissue types seems to involve different modulators to hypoxic responses. This thesis concentrated on pharmacologically modifying these complexes in order to better understand O_2 sensing in a recombinant system functionally expressing L-type Ca^{2+} channel α_{1C} subunits. It is hoped that results presented in this thesis will help shed light on O_2 sensing mechanisms and contribute to furthering knowledge in regards to how hypoxia affects ion channels. Future researchers can then utilize this knowledge to one day treat diseases that are

characterized by responses similar to those observed to be evoked during either acute or chronic hypoxia.

CHAPTER 1

The acute hypoxic response of L-type Ca^{2+} channel α_{1C} subunits during altered levels of reactive O_2 species

SUMMARY

This study investigated the involvement of reactive O_2 species (ROS) in mediating the acute hypoxic response in recombinant L-type Ca^{2+} channel α_{1C} subunits. Over the past years evidence has arisen that supports the involvement of ROS and ROS-generating complexes in the O_2 signaling pathway in a variety of O_2 sensitive cells. Furthermore, the application of ROS has been shown to alter several types of ion channels in a manner similar to that seen during acute hypoxia. Acute hypoxia inhibits Ca^{2+} currents in a variety of cells, including vascular smooth muscle cells and recombinant HEK 293 cells. Whole-cell currents were recorded in HEK 293 cells expressing functional L-type Ca^{2+} channel α_{1C} subunits, as demonstrated by application of nifedipine, an L-type Ca^{2+} channel blocker, which inhibited inward Ca^{2+} currents. Acute hypoxia inhibited Ca^{2+} currents through recombinant L-type Ca^{2+} channels in a voltage-dependent manner. Application of exogenous ROS (H_2O_2 and tert-butyl hydroperoxide) enhanced Ca^{2+} currents while dialysis or incubation with catalase inhibited Ca^{2+} currents in a manner similar to that seen during acute hypoxia. However, in catalase-dialyzed cells, Ca^{2+} currents were observed to be inhibited during acute hypoxia. In contrast, application of superoxide dismutase (SOD) inhibited Ca^{2+} currents under normoxic conditions and abrogated the hypoxic response of these channels. These

data suggested that although alterations in the levels of H_2O_2 regulate channel activity under normoxic conditions, it did not mediate the hypoxic response of these recombinant channels and that alteration in the levels of O_2^- with SOD was able to abrogate the hypoxic response. Thus, O_2^- may act as a signaling molecule in O_2 sensing. It is also possible that SOD acts as an O_2 sensor as varying the levels of this enzyme by exogenous application affected channel gating and hypoxic responses.

Next, the role of mitochondria and NADPH oxidase were examined using either knock-out models (mitochondria) or pharmacological inhibition (NADPH oxidase), respectively. Functional mitochondria were shown to be required for O_2 sensing while NADPH oxidase, a complex that forms O_2^- from O_2, was not found to be involved in the acute hypoxic response of recombinant channels. These data support a model of O_2 sensing in which O_2^-, derived from the mitochondria, are required to mediate the acute hypoxic response in L-type Ca^{2+} channel α_{1C} subunits. Further studies using inhibitors and/or producers of O_2^- to alter cellular levels need to be investigated to help support or refute this model.

INTRODUCTION

Ion channels are found in every excitable cell. Ion channel activity is closely regulated in order to carry out specific physiological functions such as neurotransmitter release, excitation-contraction, propagation of electrical signals and cell signaling. Deviation from normal channel activity can have disastrous consequences that may

ultimately lead to cell death, development of pathological disease and even organismal death. O_2 tension is a well described regulator of ion channel activity. In order for O_2 tension to regulate ion channel activity, organisms must have a way in which they can sense O_2 levels to ensure that cells and tissues receive adequate O_2 supply. They must also be able to respond to changing O_2 levels in a timely manner as prolonged periods of inadequate O_2 supply can have severe negative outcomes if careful corrective measures are not taken.

The first type of ion channel that demonstrated sensitivity to changes in O_2 levels came from K^+ channels in carotid body type I cells (Lopez-Barneo *et al.*, 1988). These channels were inhibited during exposure to hypoxia. Inhibition of K^+ channels by hypoxia in carotid body type I cells leads to cell depolarization, Ca^{2+} influx and subsequent release of neurotransmitters which relay messages to the respiratory centers of the brain to increase breathing rate/depth (Lopez-Barneo, 2003). Similar observations were made in K^+ channels of other cell types such as vascular smooth muscle cells (Weir and Archer, 1995), neuroepithelial cells (Youngson *et al.*, 1993) and central neurons (Jiang and Haddad, 1994). Further research demonstrated that other ion channels could also be regulated by hypoxia.

L-type Ca^{2+} channels are found in vascular smooth muscle and cardiac myocytes and function to control excitation-contraction coupling as well as Ca^{2+} influx into cells (Wolf *et al.*, 2003). These channels have also been shown to be regulated by hypoxia.

L-type Ca^{2+} channels in smooth muscle cells from differing parts of the vasculature decreased Ca^{2+} influx upon application of acute hypoxia as demonstrated by alterations in Ca^{2+} oscillations, which measured the amount of extracellular Ca^{2+} influx into these cells (Franco-Obregon *et al.*, 1995). This was among the first studies to demonstrate that Ca^{2+} channels could also be regulated by hypoxia. Subsequent studies have demonstrated that L-type Ca^{2+} channels are inhibited by hypoxia in various cells such as, proximal smooth muscle cells, human coronary myocytes and arteriole smooth muscle cells (Franco-Obregon and Lopez-Barneo, 1996b; Welsh *et al.*, 1998; Smani *et al.*, 2002). Not only does hypoxia inhibit L-type Ca^{2+} channels in isolated cells, it also inhibits Ca^{2+} currents in recombinant HEK 293 cells expressing L-type Ca^{2+} channel α_{1C} subunits (Fearon *et al.*, 1997). This study was important for two reasons; the first of which was that it was the first study that demonstrated that recombinant L-type Ca^{2+} channels could be reversibly inhibited by hypoxia and the second was that it demonstrated that auxiliary subunits were not required for O_2 sensing. This group later found that cysteine residues in the recombinant channel were susceptible to redox modulation, which altered channel function (Fearon *et al.,* 1999). In addition, 39 amino acid residues in the C-terminal tail of the L-type Ca^{2+} channel α_{1C} subunit were required for O_2 sensing (Fearon *et al.*, 2000). The exact mechanism of how L-type Ca^{2+} channel or other O_2 sensitive channels are able to sense and response to altered O_2 levels has not yet been elucidated.

Many models as to how cells are able to "sense" changes in Po_2 levels have been postulated over the past years. These models include the involvement of complexes, such

as NADPH oxidase and mitochondria, or complex by-products, such as ROS that are directly affected by changes in O_2 levels. The NADPH oxidase model of O_2 sensing proposes that decreased levels of O_2 alters the levels of ROS produced by NADPH oxidase which then alters the redox state of the cell. NADPH oxidase is a multi-subunit complex that generates O_2^- from O_2, which is then dismutated to H_2O_2 by superoxide dismutase (SOD) (Chandel and Schumacher, 2000; Jones *et al.*, 2000; Lopez-Barneo *et al.*, 2004). Evidence has also arisen that has supported NADPH oxidase as the O_2 sensor. In neuroepithelial body cells of knock-out mice that lacked the enzymatic $gp91^{phox}$ subunit of NADPH oxidase, which catalyzes the formation of O_2^-, hypoxic inhibition of K^+ currents was abolished (Fu *et al.*, 2000). Hypoxic inhibition of K^+ channels occurred in cells of mice that had not been genetically modified so it was thought that NADPH oxidase acted as the O_2 sensor in these cells. Other studies have refuted the involvement of NADPH oxidase in O_2 sensing (O'Kelly *et al.,* 2001) and other complexes have been proposed to be involved in O_2 sensing.

Mitochondria are excellent O_2 sensor candidates as they consume most of the O_2 used by a cell and would therefore be directly affected by changing O_2 levels. Previous studies investigating the role of mitochondria in O_2 sensing have demonstrated that application of mitochondrial inhibitors alter ion channel activity in manner similar to that seen during hypoxia (Mills and Jobsis, 1972; Mulligan, 1981; Biscoe and Duchen, 1989). The presence of functional mitochondria is also important in O_2 sensing as cells that lack functional mitochondria (ρ^0 cells) failed to respond to hypoxia (Waypa *et al.*, 2001).

Other studies have reported that mitochondria are not essential for O_2 sensing (Searle *et al.*, 2002) and as such further research has concentrated investigation on the involvement of molecules derived from mitochondria and NADPH oxidase.

An alternative theory in O_2 sensing proposes the involvement of mitochondrial or NADPH oxidase-derived ROS, a natural byproduct of cells that are thought to be directly affected by O_2 levels. This theory postulates that decreased availability of O_2 decreases the amount of ROS generated by NADPH oxidase and mitochondrial ETC complexes such that there is an increase in the ratio of reduced compounds in the cell that may modify channel residues and alter channel activity. This theory has come about by previous findings that measured ROS production during hypoxia. Archer (1993) found ROS production decreased during hypoxia and that this decrease was associated with an increase in pulmonary arterial pressure, a characteristic hypoxic response. Further studies have shown that K^+ channel and L-type Ca^{2+} channel activity can be altered with varying ROS levels (Wang *et al.*, 1996; Thomas *et al.*, 1998; Akaishi *et al.*, 2004; Tang *et al.*, 2004) and that alterations in activity strongly resemble responses seen during hypoxia. Recombinant L-type Ca^{2+} channels are known to be inhibited by hypoxia and redox modulation (Fearon *et al.*, 1999) in a similar manner to K^+ channels. It is thought that redox modulation of channel residues may alter channel function. In addition to this, hypoxia is thought to alter the redox state of the cell by diminishing levels of O_2^- and H_2O_2 generated during normoxia. Thus, it was of interest to determine how alterations in ROS levels in recombinant HEK 293 cells affected L-type Ca^{2+} channels in response to

acute hypoxia. In this study, I altered ROS levels either by exogenous application of ROS species, such as H_2O_2, or by altering levels of enzymes that catalyze reactions that form ROS, such as catalase and SOD. I also investigated the involvement of mitochondria by comparing the hypoxic response between cells lacking functional mitochondrial DNA (ρ^0) and wild-type (ρ^+) cells, in addition to examining the acute hypoxic response of cells during inhibition of NADPH oxidase by NADPH oxidase inhibitors, such as DPI and PAO. The results indicate that mitochondrial derived O_2^- is an essential part of O_2 sensing in recombinant L-type Ca^{2+} channel α_{1C} subunits.

METHODS AND MATERIALS

Stable Transfection of HEK 293 cells

All experiments were carried out using human embryonic kidney (HEK) 293 cells stably expressing human cardiac L-type Ca^{2+} channel α_{1C} subunits (Schultz *et al.*, 1993). The α_{1C} subunit cDNA clone was a kind gift from Dr. Gyula Varadi (University of Cincinatti). All stable transfections were performed by Mr. Stephen Brown (Research Technician) as follows. Wild-type HEK 293 cells were initially transfected with 3 μg pCDNA3.1-α_{1C} using ExGen 500 (Fermentas, Burlington, ON, Canada) according to the manufacturer's instructions. Transfection took place after splitting the cells the previous day to approximately 60 % confluency. The medium was replaced three days post-

transfection with medium containing 400 mg/ml G418 (Invitrogen). Individual colonies were visualized using an inverted microscope (Zeiss) and phase-contrast objectives. Visualization of colonies took place after two weeks, during which time selection was applied continuously. Colonies were then picked and seeded in wells of a 96-well plate and allowed to grow to confluency. Once confluency was reached, the colonies were transferred to 35 mm dishes for further culture and for examination of Ca^{2+} currents. 60 clones were screened and were positive for Ca^{2+} channel activity. A single clone of this >50 % was selected for further study based on the number of cells within the clone expressing substantial current. This clone was then sub-cloned to ensure a pure population of stably transfected cells. G418 selection was continued throughout the cloning process and in all subsequent sub-culturing.

Culture of HEK 293 cells

Cells were grown in minimal essential medium (MEM) with Earle's salts and L-glutamine (Gibco, Paisley, UK) containing 9 % (v/v) fetal calf serum (Globepharm, Esher, Surrey, UK), 1 % (v/v) non-essential amino acids, gentamicin (50 mg/L), 10,000 U L^{-1} penicillin G, 10 mg/L streptomycin, 0.25 mg/L amphotericin and 400 mg/L G418 (all Gibco, Mississauga, ON). Cells were incubated at 37 °C in a NAPCO CO_2 6000 incubator in an atmosphere comprised of 95 % air and 5% CO_2. Cells were cultured in 35 mm dishes and split three times a week at a ratio of 1:4. Cells were split at least 24 h

prior to electrophysiological experimentation and kept in the incubator until at least 5 min before experimentation was to begin.

ρ^0 Cell Culture

The mitochondrial DNA depleted cell line (ρ^0 cells) was generated from HEK 293 cells stably expressing recombinant L-type Ca^{2+} channel α_{1C} subunits (see above). These cells were initially grown in MEM (as described above) supplemented with ethidium bromide (500 ng/ml; Sigma), pyruvate (1 mM), and uridine (50 μg/ml). The ρ^0 mitochondrial DNA levels were examined monthly and compared to cells grown in media in the absence of ethidium bromide, pyruvate and uridine (wild-type cells). The mitochondrial DNA levels in the ρ^0 cells after the first, second and third months of growth in ethidium bromide were approximately 60 %, 40 %, and 2 % of wild-type levels respectively. Also, the concentration of ethidium bromide was increased after the first and second months to 1 μg/ml and 2 μg/ml respectively. MtDNA levels were examined as follows; DNA was harvested from the cells using the DNeasy Tissue Kit (Qiagen), and was analyzed and quantitated by spectrophotometry (260/280 nm). Equal amounts of DNA from the ρ^0 cells and wild-type cells were subjected to quantitative PCR using the Brilliant SYBR green QPCR Master Mix (Stratagene), the Mx3000P QPCR machine (Stratagene) and human specific primers pairs for mtDNA: upstream 5'-CCT AGG GAT AAC AGC GCA AT-3' and downstream 5'-TAG AAG AGC GAT GGT GAG AG-3'

and β-actin: upstream 5'-TGG CCG GGA CCT GAC TGA CTA C-3' downstream 5'-CGT GGC CAT CTC TTG CTC GAA G-3' as described previously (Searle *et al.*, 2002).

Cell Passaging

To passage cells, dishes were removed from the incubator and media was removed by suction. Cells were washed in sterile PBS, after which 0.7 mL of 10 mM trypsin-EDTA (Gibco) was added to the dish to disrupt cell adhesion from the plate bottom as well as deadhere the cells from each other. 2 mL of pre-warmed media was added to each new dish. After 45 s, the trypsinated dish containing cells was pipetted up and down back onto the plate bottom to remove any residual cells attached to the bottom of the dish. Suction was again applied with a 1 mL pipette to remove the trypsinated cells and 2-3 drops of this solution was placed in a new dish containing 2 mL of pre-warmed media. This was done to 3-5 dishes after which the media was swirled in each dish by hand to ensure the cells would spread out evenly on the dish bottom. Dish lids were marked as to cell type, dated accordingly and placed back in the incubator.

Electrophysiology

Patch-Clamp Electrode Construction

Patch electrodes were constructed from 1.5 mm diameter borosilicate glass capillary tubes (1B150F-4; World Precision Instruments, Sarasota, FL). Patch electrode

were fabricated in a vertical electrode puller (PC-10, Narishige Co. Ltd., Tokyo, Japan) at room temperature (~ 23 °C). Final resistance of the electrodes was 3.0-6.5 MΩ. Patch electrode tips were polished using a glass fire polisher (Micro Forge MF-830, Narishige Co. Ltd. Tokyo, Japan) and placed in a 15 cm dish with a strip of plasticine to immobilize the electrodes and protect them from breakage and dust.

Extracellular and Intracellular Solutions

The extracellular perfusate consisted of (in mM): NaCl, 95; CsCl, 5; $MgCl_2$, 0.6; BaCl, 20; Hepes, 5; D-glucose, 10; and TEA-Cl, 20 (20-24 °C, pH adjusted to 7.4 with NaOH). Intracellular solution used in whole cell recordings consisted of in mM): CsCl, 120; TEA-Cl, 20; MgCl, 2; EGTA, 10; Hepes, 10 and ATP, 2 (pH adjusted to 7.2 with CsOH).

Experimental Apparatus

All experiments were performed using the whole-cell patch-clamp technique (Hamill *et al.*, 1981). Electrophysiological recordings were made in a continuously perfused recording chamber (approximately 3-5 mL/min) that was mounted on the stage of a Nikon TE2000-U inverted microscope (Nikon, Canada). The perfusion system consisted of four perfusion reservoirs made from 60 mL syringe tubes and connected via three-way Luer lock valves. A three-way valve at the output of the perfusion apparatus

controlled the rate at which the chamber was perfused; perfusate flow itself was driven by gravity. The perfusion apparatus and the chamber were connected via Tygon tubing (Tygon, Saint-Gobain Performance Plastics Corporation, Akron, Ohio, USA), through which the perfusate was allowed to flow. This tubing is non-contaminating, non-oxidizing and is less gas permeable than rubber tubing. It was of importance to use gas impermeable tubing to ensure perfusate O_2 levels remained constant during the course of the experiment. Perfusate was removed from the recording chamber via a metal tube bent at a 45° angle and connected to 1.5 mm diameter silicone tubing, which drained into a Buchner flask. Suction was provided by water flowing through a tap connected to an aspirator. Perfusate was removed from the recording chamber of at a rate relative to the input rate from the perfusion apparatus such that there was a constant fluid level in the recording chamber during recordings. Each perfusion apparatus tube contained either extracellular solution, extracellular solution bubbled with N_2 gas (hypoxic solution), extracellular solution containing a drug or extracellular solution containing a drug and bubbled with N_2 gas.

Patch electrodes were filled with intracellular solution via a blunt-needled 5 mL syringe containing intracellular fluid. The intracellular solution was placed on ice while recording to prevent breakdown of ATP. Filled electrodes were then placed in an electrode holder (Axon Instruments, CV-7B), which contained a 4 cm long chlorided silver wire to form an electrical connection between the patch-amplifier and the intracellular solution. The holder was attached to an amplifier headstage and a patch-

clamp amplifier (Multiclamp 700 Amplifier, Axon Instruments). A silver/silver chloride pellet connected to the headstage was placed in the recording chamber to provide grounding. Seal formation between the electrode tip and the cell was accomplished via suction through the electrode. This was made possible by a portion of silicone tubing that was used to connect the electrode holder to a 1 mL pipette tip. Similar suction was applied to rupture the membrane in order to make whole-cell patch clamp recordings.

The entire apparatus was surrounded by a Faraday cage to reduce electrical interference and was mounted on an anti-vibration table (Technical Manufacturing Corporation, Peabody, MA, USA) to reduce mechanical interference. Cells were viewed at 400x magnification and all electrophysiological recordings were made at room temperature (24 °C ± 2 °C).

Experimental Technique and Methodology – Whole-cell patch-clamp recording

After the patch electrode was placed in the electrode holder, it was then guided into the recording chamber using the coarse manipulator (Burleigh TS-5000-150). Adjustments were made using the coarse manipulator such that the electrode was visible through the eyepiece of the microscope. The patch electrode was slowly advanced toward the cell first using the coarse manipulator to place the electrode tip in proximity of the desired cell and then using the fine micromanipulator (Burleigh PCS-PS60). Electrode resistance was monitored by continuous application of a 5 mV voltage step.

Offset potential was zeroed as necessary. When series resistance increased as the electrode touched the cell surface, slight suction was applied through the electrode holder. Seal formation was seen as a sudden increase in resistance, visualized as a decrease in current amplitude elicited by the 5 mV voltage step. The "Cp-Fast: Auto" button was then pushed when the seal reached a resistance of 1 GΩ or greater and the holding potential of the cell was changed from 0 mV to -80 mV. Pushing the "Cp-fast: Auto" button eliminates the transients caused by the capacitance of the patch electrode. Further weak suction was applied until the membrane ruptured as indicated by appearance of large capacitative transients. These transients were eliminated by pressing the "whole-cell: Auto" button on the Multiclamp amplifier, which compensates for the whole-cell capacitance transient. At this time, voltage-clamp protocols were executed as described below.

Experimental Protocols

Voltage-clamp protocols were performed using a Multiclamp 700 Amplifier in combination with a Digidata 1322A interface and pCLAMP software, version 9 (Axon Instruments).

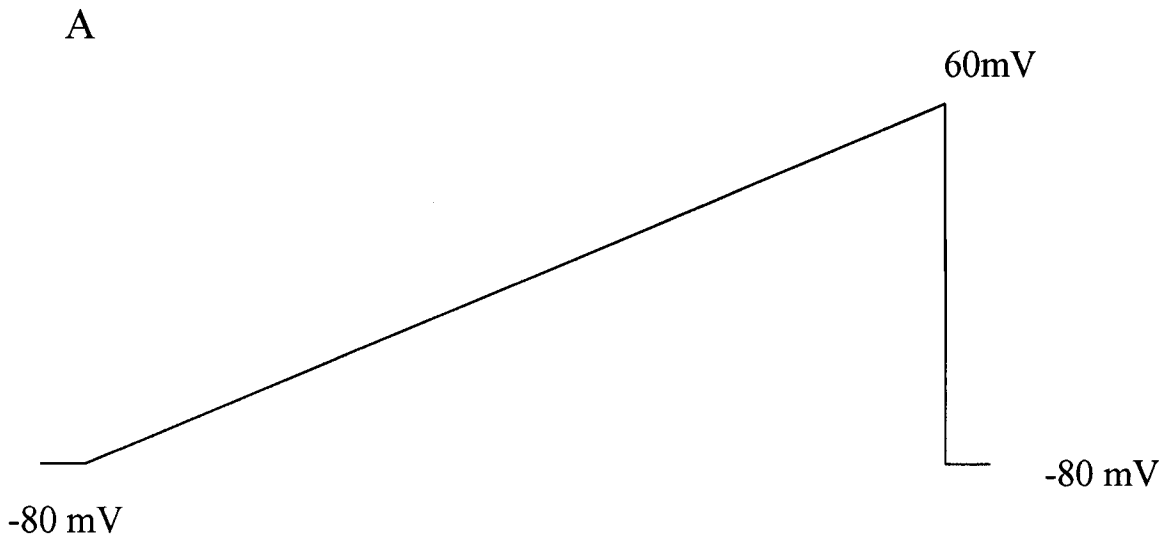
Voltage Clamp

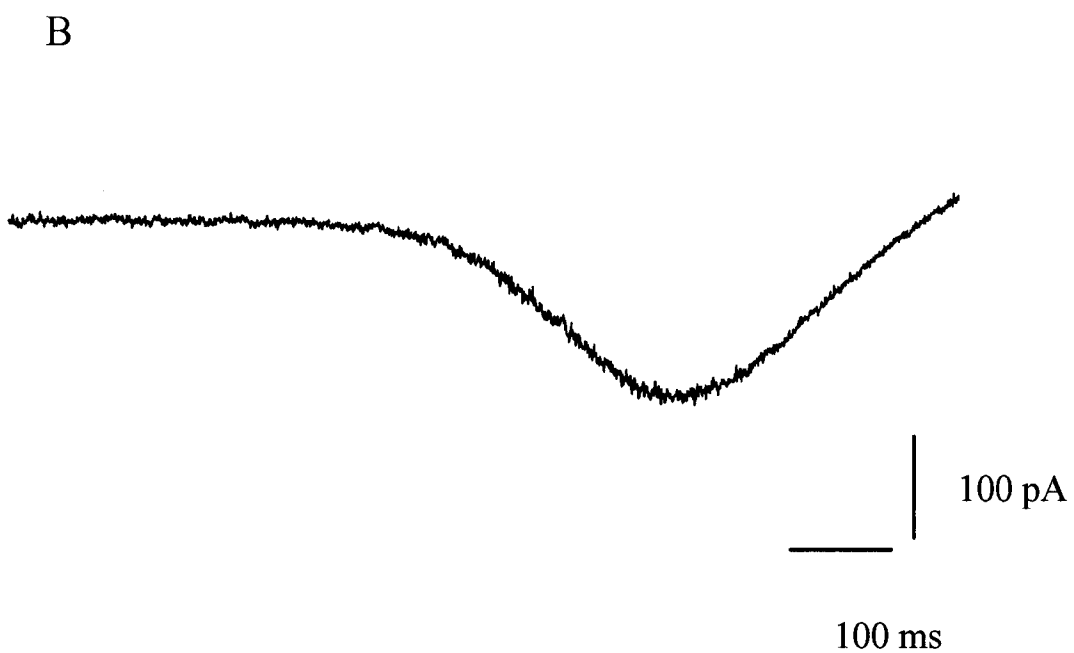
Cells were always held at a potential of -80 mV prior to time-series, ramped voltage or stepped voltage recordings. To determine if a cell exhibited current, the cell was subjected to electrical stimulation by a voltage ramp protocol (see Figure 3). Once this was determined, baseline recordings were made until current reached a constant amplitude, prior to introducing test solutions into the recording chamber. Test solutions were allowed to reach equilibrium before switching to another solution. The time-series protocol stimulated cells for 100 ms to the required potential and recorded the current evoked by that stimulation. This was repeated every 10 s. The I-V protocol stepped from -80 mV to 70 mV in 10 mV increments at a frequency of 1 Hz. The ramp-voltage protocol varied the membrane potential linearly from – 100 mV to + 80 mV over a period of 1 s and recorded the evoked current at a frequency of 1 Hz.

Recording Cell Currents and Data Analysis

Whole-cell current traces were filtered at 5 kHz, digitized at 10 kHz and stored on a computer for later analysis. Capacitative transients were minimized by analogue means and corrections for leak current was completed during data analysis by appropriate scaling and subtraction of the average leak current evoked by hyperpolarizing and depolarizing steps (≤10 mV). Data were examined offline with Clampfit 9.0 software or Microsoft Excel software and graphing of the data was performed using the Origin

Figure 3: Example voltage ramp protocol used to determine whether recombinant HEK 293 cells expressing L-type Ca^{2+} channel α_{1C} subunits exhibited Ca^{2+} currents. **A** command voltage (mV) was varied linearly from a starting voltage of -80 mV to +60 mV over a period of 1 s. At a command voltage of -80 mV, Ca^{2+} currents in recombinant HEK 293 cells expressing L-type Ca^{2+} channel α_{1C} subunits are non-conducting until command voltage reaches approximately -40 mV, at which point the channels open and inward Ca^{2+} currents ensue, as shown in **B**.


A
60mV
-80 mV
-80 mV


B
100 pA
100 ms

software program (Microcal). Results are expressed as means (± S.E.M). To evaluate statistical differences in data obtained from paired samples (in which the before and after current measurements were recorded from the same cell), statistical comparison was performed using a paired Student's *t*-test. Statistical differences in unpaired samples (in which the before and after measurements were taken in two separate populations of cells), were made with an unpaired Student's *t*-test. Both tests used were two tailed, as it was possible for the calculated mean value to either increase or decrease in the presence of experimental drug solutions used in this study. Values were considered significant if the probability value (P) was less than 0.05.

Acute Hypoxia Methodology

Acute hypoxia was produced by bubbling the extracellular perfusate with N_2 gas (100 %) in a semi-sealed reservoir tube for 30 min prior to application. Sealing was provided by stretching parafilm around the top of the reservoir until a tight seal was obtained. By perforating the Parafilm with Tygon tubing, nitrogen gas was introduced to the reservoir perfusate. Gas-impermeable Tygon tubing was also used for every tubing part of the perfusion system to ensure that the degree of hypoxia remained constant as the extracellular perfusate traveled from the reservoir to the recording chamber.

Measuring O_2 tension in the recording chamber

Bath Po_2 was measured using a depolarized (-600 mV) carbon fibre electrode, and was always stable at ~20 mmHg within 30-45 s of exchanging solution . The pH of the extracellular perfusate was not altered by bubbling with N_2 gas. The depolarized carbon fibre electrode measured reducible elements in solution. Baseline current corresponds to an O_2 tension of 150 mmHg while zero current is obtained in an anoxic perfusate. When hypoxic extracellular perfusate was introduced into the recording chamber, the amount of current decreased proportional to the amount of O_2 in the perfusate. The O_2 tension in solution was then mathematically derived.

Drug and Enzyme solutions

Drug and enzyme solutions were prepared by adding and/or dissolving the drug/enzyme into the intracellular or extracellular perfusate. For some experiments, it was included as part of the culture for incubation over a 24 h period. pH was adjusted as necessary. All drugs/enzymes used in this thesis were obtained from Sigma Aldrich.

Nifedipine: 6 μL of 20 mM stock solution nifedipine in DMSO was injected into a reservoir tube containing 60 mL of extracellular fluid for a final concentration of 2 μM. This solution was allowed to perfuse the recording chamber after control currents had been recorded.

Hydrogen peroxide: 2.3 μL of 30 % hydrogen peroxide was added to 30 ml of extracellular perfusate 30 seconds before perfusion with this solution was to occur. Due to its highly unstable nature, H_2O_2 was kept on ice (at 4 °C) prior to its addition to the extracellular perfusate. Final concentration of the solution for all experiments was 100 μM.

Tert-Butyl hydroperoxide: 1.0 μL of 70 % aqueous solution tert-butyl hydroperoxide was added to 30 ml of extracellular solution (final concentration of 50 μM) immediately before perfusion. To avoid breakdown, tert-butyl hydroperoxide was also kept at 4 °C and wrapped with aluminum foil due to its light and temperature sensitivity.

Catalase (intracellular): Catalase was added to the intracellular solution at a concentration of 1000 units/ml (5 μL of 33 mg protein/mL crystalline suspension in H_2O stock in 2 ml). The solution was mixed well, then loaded into a syringe with a blunted needle at the end and injected into the patch electrode. Intracellular solution was kept on ice to prevent breakdown of catalase. Cells were dialyzed with this solution for a minimum of 15 min to allow the drug to enter the cell. Calculations (Pusch and Neher, 1988) demonstrate that 15 min is sufficient time to allow catalase to permeate the cell given that the MW of catalase is 60 kDa (Sundaresan *et al.*, 1995) and the resistance of the patch pipette was less than 10 MΩ.

Catalase (extracellular) – 2.5 μL of 33 mg protein/mL crystalline suspension in H_2O catalase was added to 2 ml of culture medium to give a final concentration of 500 units/mL. This was then used to incubate the cells for 24 h prior to recordings. In addition, the extracellular fluid also included catalase at a concentration of 500 units/mL (250 μL of catalase for every 200 mL of extracellular fluid). This was performed just prior to pouring the extracellular perfusate into the reservoir tube.

Superoxide dismutase (SOD) – 2.5 μL was added to 2 ml of intracellular solution for a final concentration of 250 units/ml. This was injected into the patch electrodes prior to recordings and was kept on ice when not being used. 15 min was given to allow the SOD to dialyze into the cell. As determined by the calculations by Pusch and Neher (1988), the MW of SOD being 32 kDa and the end resistance of the electrodes being <10 MΩ, this was enough time to allow SOD to equilibrate with the cytoplasm.

Diphenylene iodonium (DPI) – 250 μL of 10 mM stock solution of DPI in DMSO was added to 250 ml of extracellular perfusate (final concentration was 10 μM). This solution was stored in a 250 ml flask and added to a reservoir tube prior to perfusion.

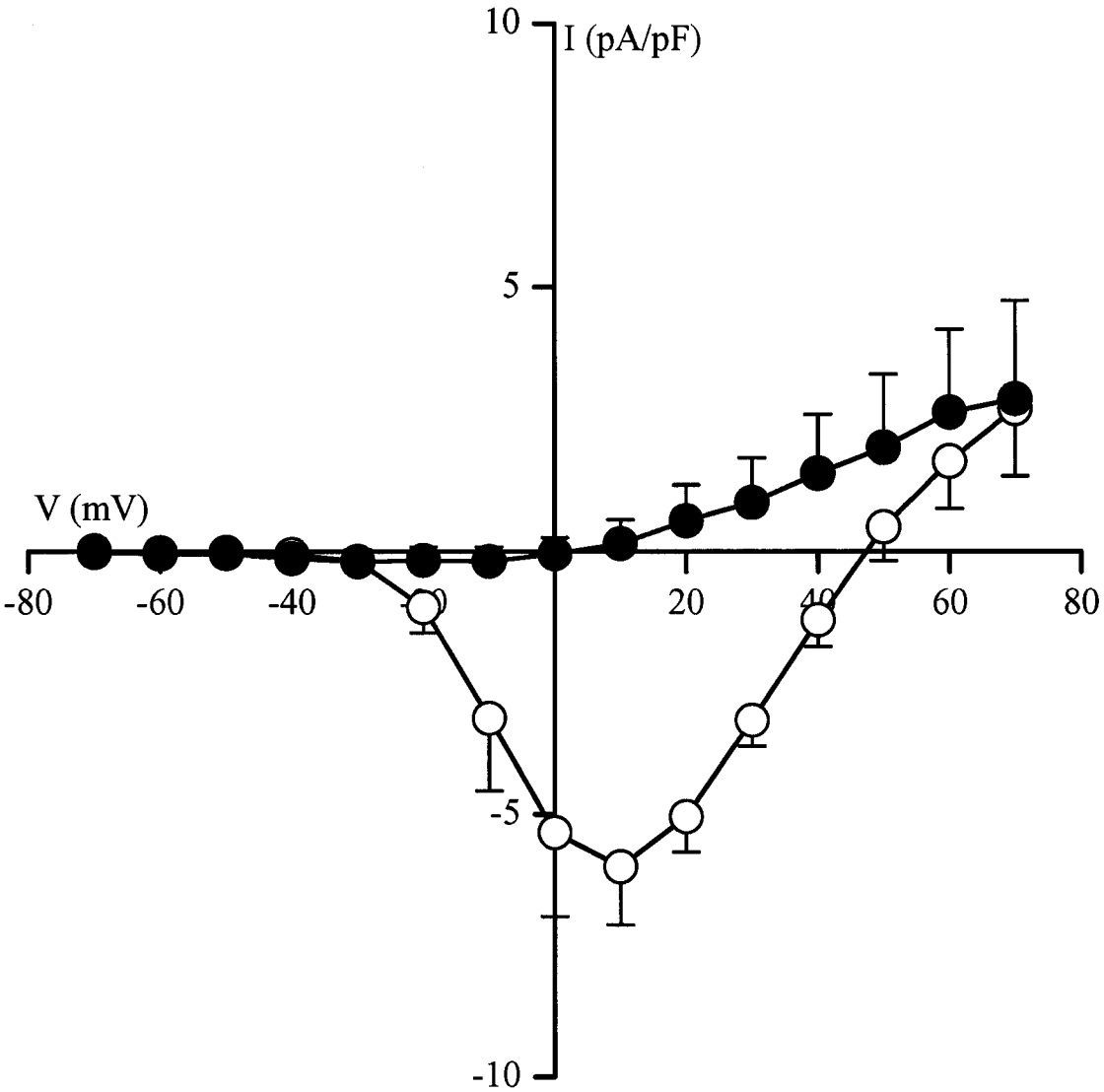
Phenylarsine Oxide (PAO) – 125 μL of 10 mM stock solution of PAO in DMSO was added to 250 ml extracellular perfusate (final concentration was 5 μM). This solution was stored in a 250 ml flask and added to a reservoir tube prior to perfusion.

RESULTS

HEK 293 cells express functional L-type Ca^{2+} channel α_{1C} subunits

To determine if stably-transfected HEK 293 cells expressed functional L-type Ca^{2+} channel α_{1C} subunits the L-type Ca^{2+} channel blocker nifedipine was applied to cells during whole-cell electrophysiological recordings. Nifedipine binds to the α_{1C} subunit of L-type Ca^{2+} channels of cardiac and vascular smooth muscle (Shepard and Stump, 1999). When channel openings are stimulated by depolarization, no current is evoked (Hille, 2001). As demonstrated in Figure 4, application of 2 μM nifedipine inhibited Ca^{2+} channel currents at all test potentials examined. Inhibition was not voltage-dependent, such that currents were fully inhibited at all test potentials examined. For example, at a test potential of +10 mV (the potential at which control currents were maximal), Ca^{2+} currents in cells prior to the application of 2 μM nifedipine were -6.00 ± 1.11 pA/pF ($n = 5$), and were reduced to 0.15 ± 0.45 pA/pF during exposure to nifedipine ($n = 5$; $P < 0.05$, paired Student's *t*-test). At a test potential where Ca^{2+} currents were more depolarized to those at which maximal currents were evoked (+40 mV), inward Ca^{2+} channel currents in cells prior to application of 2 μM nifedipine were -1.31 ± 0.51 pA/pF ($n = 5$) and 1.48 ± 1.10 pA/pF after nifedipine exposure ($n = 5$; $P > 0.05$, paired Student's *t*-test). Since Ca^{2+} currents could be evoked prior to but not during application of nifedipine, these results demonstrate that L-type Ca^{2+} channel α_{1C} subunits were functionally expressed in HEK 293 cells.

Figure 4: Effect of 2 μM nifedipine on Ca^{2+} currents through recombinant L-type Ca^{2+} channel α_{1C} subunits. Currents were evoked (by step depolarizing cells for 100 ms to indicated test potential at a frequency of 1 Hz) in the absence (○, n = 6) or presence (●, n = 6) of nifedipine. Current density (I, pA/pF) was obtained by dividing the evoked current by the cell capacitance. Data are presented as means (± S.E.M).


10
I (pA/pF)
5
V (mV)
-80
-60
-40
20
60
80
-5
-10

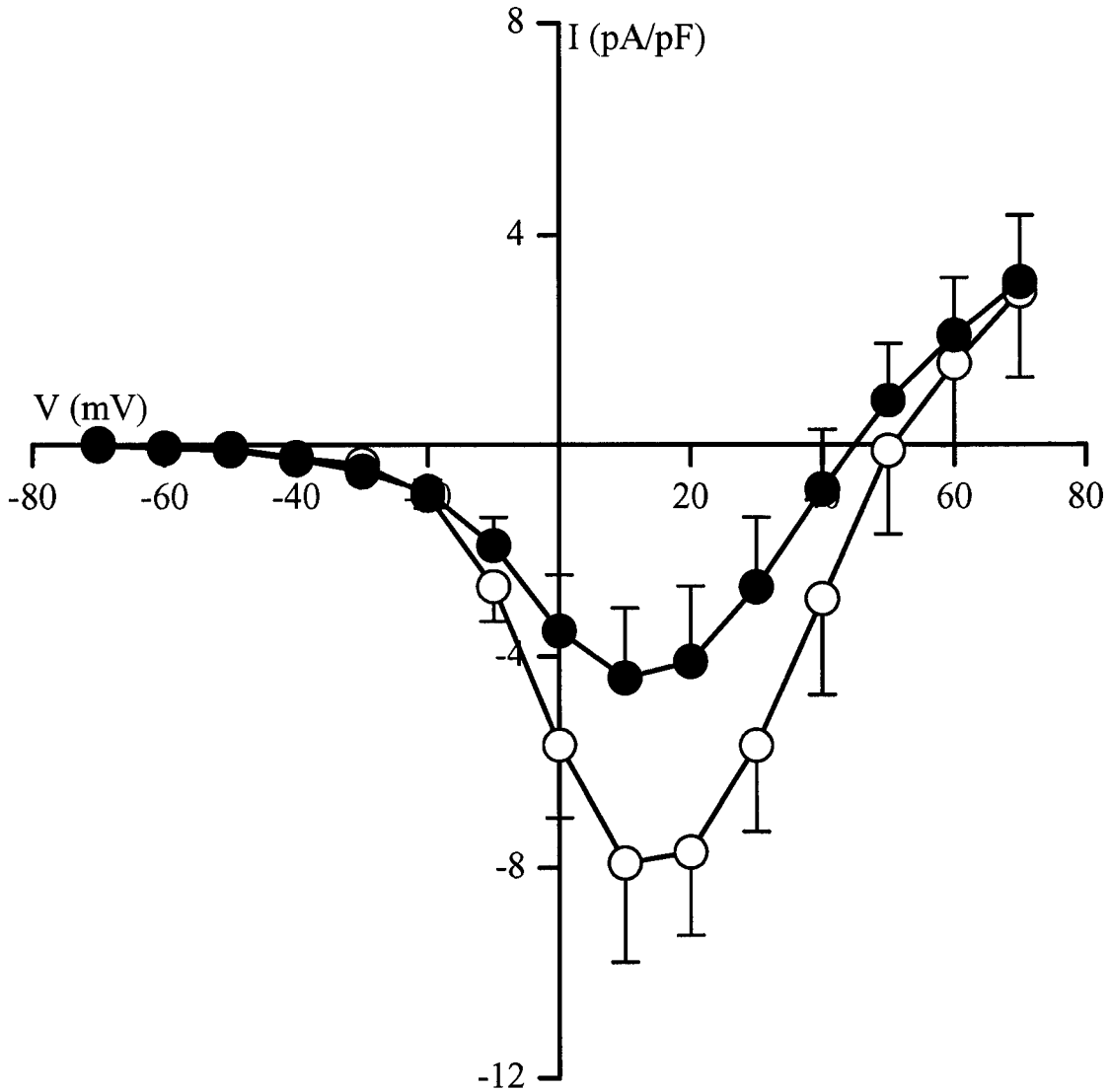
Acute hypoxia inhibited Ca^{2+} currents through L-type Ca^{2+} channel α_{1C} subunits

As described above, HEK 293 expressed functional L-type Ca^{2+} channel α_{1C} subunits. Given the O_2 sensitivity of these channels (Fearon *et al.*, 1997) and given my proposed use of this newly-created cell line to examine O_2 sensing mechanisms, it was important to demonstrate the response of these subunits to acute hypoxia. Cells were exposed to hypoxic extracellular perfusate (Po_2 ~ 20 mmHg) while recording whole-cell Ca^{2+} channel currents. A current-voltage (I-V) protocol was used to record currents at various test potentials before and during application of acute hypoxia. The mean I-V relationships demonstrate the inhibition of Ca^{2+} current during acute hypoxia (Figure 5). For example, at +10 mV the mean (± S.E.M.) current was -7.92 ± 1.88 pA/pF in control cells ($n = 5$) and was -4.41 ± 1.32 pA/pF in cells exposed to acute hypoxia ($n = 5$; $P < 0.05$, paired Student's *t*-test). Thus, acute hypoxia inhibited channel activity such that there was a decrease in Ca^{2+} current during acute hypoxia.

Exogenous reactive O_2 species enhanced Ca^{2+} currents but did not modulate the response to acute hypoxia

Reactive O_2 species (ROS) have been proposed as a regulator of K^+ channel activity in porcine renal artery, canine tracheal, rat brain, ductus arteriosus smooth muscle and skeletal muscle cells (Janssen *et al.*, 2000; Shin *et al.*, 2000; Reeve *et al.*,

Figure 5: Acute hypoxia inhibited Ca^{2+} current through recombinant L-type Ca^{2+} channel α_{1C} subunits. Current density-voltage relationships are shown for cells prior to (○, n = 5) and during exposure to acute hypoxia (●, n = 5) (P_{O_2} ~ 20 mmHg). Currents were evoked by step depolarizing cells for 100 ms to indicated test potential at a frequency of 1 Hz. Current density (I, pA/pF) was obtained by dividing the evoked current by the cell capacitance. Data are presented as means (± S.E.M).


8
I (pA/pF)
4
V (mV)
-80
-60
-40
20
60
80
-8
-12

2001a; Soto *et al.*, 2002; Brakemeier *et al.*, 2003) as well as being a proposed mediator of acute hypoxic signaling to K^+ channels of vascular smooth muscle (Michelakis *et al.*, 2002a). The aim of this study was to examine whether the activity of recombinant L-type Ca^{2+} channels was modulated by increased levels of exogenously-applied H_2O_2 (a form ROS), and further to examine whether altered H_2O_2 levels mediates the acute hypoxic response. Bath-application of 100 μM H_2O_2 enhanced Ca^{2+} current density in a voltage-dependent manner, such that maximal enhancement occurred at test potentials more negative than those at which currents were maximal (Figure 6). The magnitude of current enhancement due to H_2O_2 was decreased at test potentials both depolarized and hyperpolarized to those which evoked maximal currents. For example, at a test potential of 0 mV, control currents were -4.3 ± 1.1 pA/pF, a value which increased to -7.4 ± 1.2 pA/pF upon exposure to H_2O_2 ($n = 5$; $P < 0.05$, paired Student's *t*-test). At a test potential of +30 mV control currents were -4.7 ± 1.3 pA/pF, a mean value not significantly different to that seen during exposure to H_2O_2 (-4.0 ± 0.3 pA/pF; $P > 0.05$, paired Student's *t*-test). Similarly, Figure 7 demonstrates that bath applied 50 μM tert-butyl hydroperoxide (an analog of H_2O_2) also enhanced Ca^{2+} currents in a voltage-dependent manner. For example at 0 mV, Ca^{2+} currents were -6.4 ± 1.23 pA/pF prior to the addition of tert-butyl hydroperoxide, and were -8.7 ± 1.01 pA/pF upon addition of the ROS ($n = 5$, $P < 0.05$, paired Student's *t*-test). Contrastingly, at a test potential of +30 mV, Ca^{2+} currents were -6.81 ± 1.17 pA/pF (control) and -5.85 ± 1.32 pA/pF (tert-butyl hydroperoxide; $n = 5$; $P > 0.05$, paired Student's *t*-test). Taken

Figure 6: Effect of 100 μM H_2O_2 on Ca^{2+} currents through L-type Ca^{2+} channel α_{1C} subunits. Current density-voltage relationships are shown for cells prior to (○, n = 5) and during exposure to 100 μM H_2O_2 (●, n = 5). Each point shows the mean (± S.E.M) peak current evoked at various test potentials when step depolarizing the cells to the indicated test potential for 100 ms from a holding potential of −80 mV. 100 μM H_2O_2 enhanced Ca^{2+} currents in a voltage-dependent manner such that the largest current enhancement occurred before maximal current was reached (at 0 mV). Current density (I, pA/pF) was obtained by dividing the evoked current by the cell capacitance.

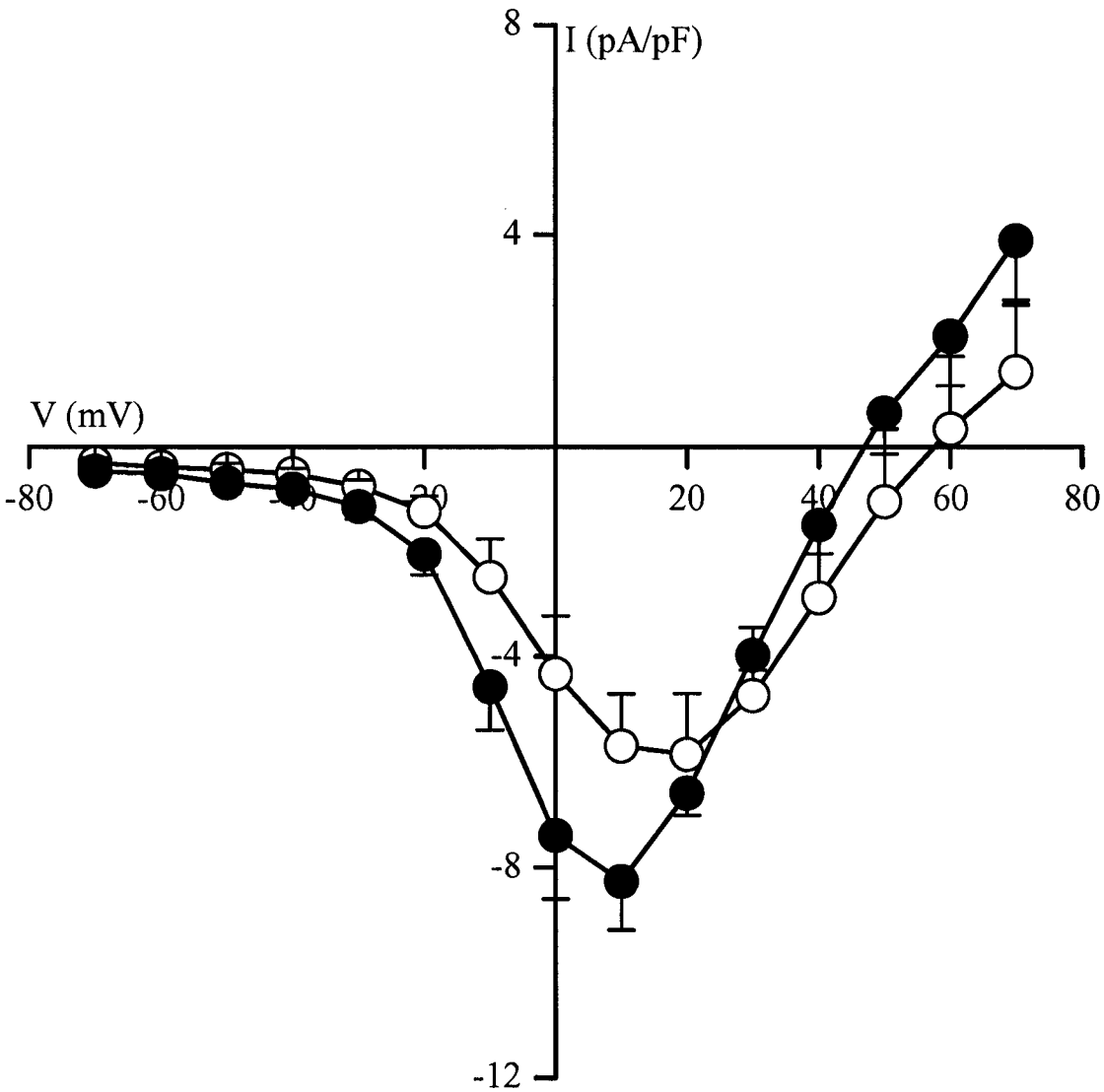
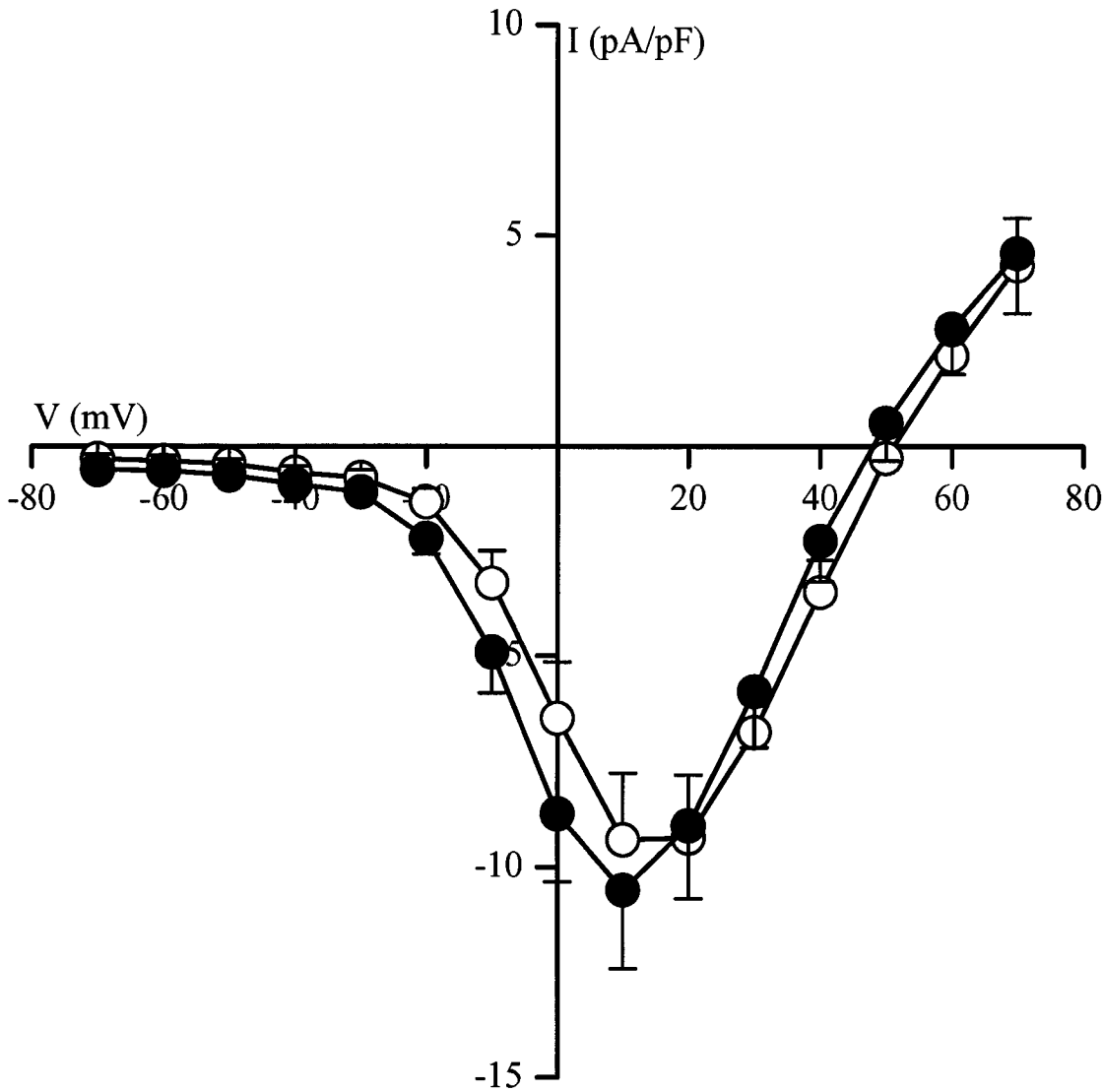

8
I (pA/pF)
4
V (mV)
-80
-60
20
40
60
80
-4
-8
-12

Figure 7: Effects of 50 μM tert-butyl hydroperoxide on Ca^{2+} currents through recombinant L-type Ca^{2+} channel α_{1C} subunits. Mean (± S.E.M) current density-voltage relationships were obtained under control conditions (○, n = 6) and during application of 50 μM tert-butyl hydroperoxide (●, n = 6). Effects were voltage-dependent such that maximal enhancement of currents occurred at test potentials more hyperpolarized than those at which currents were maximal. At values above which currents were maximal, there was no significant effect of tert-butyl hydroperoxide on Ca^{2+} currents. Currents were evoked by step depolarizing cells for 100 ms to indicated test potential at a frequency of 1 Hz. Current density (I, pA/pF) was obtained by dividing the evoked current by the cell capacitance.


10
I (pA/pF)
5
V (mV)
-80
-60
20
40
60
80
-10
-15

together, these data demonstrate that H_2O_2 enhanced recombinant Ca^{2+} channel currents in a voltage-dependent manner.

In order to investigate whether H_2O_2 mediated the acute hypoxic response of recombinant L-type Ca^{2+} channels, cells were dialyzed with catalase to reduce endogenous H_2O_2 levels, and the effects of hypoxia were examined. Cells were dialyzed for 15 min prior to electrophysiological recording either with catalase-free intracellular solution or intracellular solution containing 1000 units/ml of catalase. Figure 8 shows that catalase reduced Ca^{2+} current through the recombinant channels. This reduction in current was moderately voltage-dependent such that the greatest reduction in current occurred at potentials slightly more negative to test potentials at which maximal currents were evoked. For example at +10 mV, mean (± S.E.M.) current amplitudes were -4.80 ± 1.01 pA/pF in control cells (n = 5) and were -3.2 ± 0.87 pA/pF in cells dialyzed with catalase (n = 5) ($P < 0.05$, unpaired Student's *t*-test). This finding was confirmed by the exogenous application of 500 units/ml catalase. Catalase was added to the extracellular media in which the cells were incubated for 24 h prior to electrophysiological recording. In addition to this, 500 units/ml catalase was added to the extracellular perfusate used to bathe cells in the recording chamber during recordings, to ensure H_2O_2 levels remained reduced. As illustrated in Figure 9, incubation with catalase reduced Ca^{2+} currents in a moderately voltage-dependent manner such that maximal inhibition occurred at test potentials at which maximal currents were evoked.

Figure 8: Catalase inhibited Ca^{2+} currents through recombinant L-type Ca^{2+} channel α_{1C} subunits. Mean (± S.E.M) current density-voltage relationships obtained from cells dialyzed via the patch pipette with catalase free intracellular solution (○, n = 6) for 15 min and cells dialyzed with 1000 units/ml of catalase (●, n = 6). Currents were evoked by step depolarizing cells for 100 ms to indicated test potential at a frequency of 1 Hz. Current density (I, pA/pF) was obtained by dividing the evoked current by the cell capacitance.

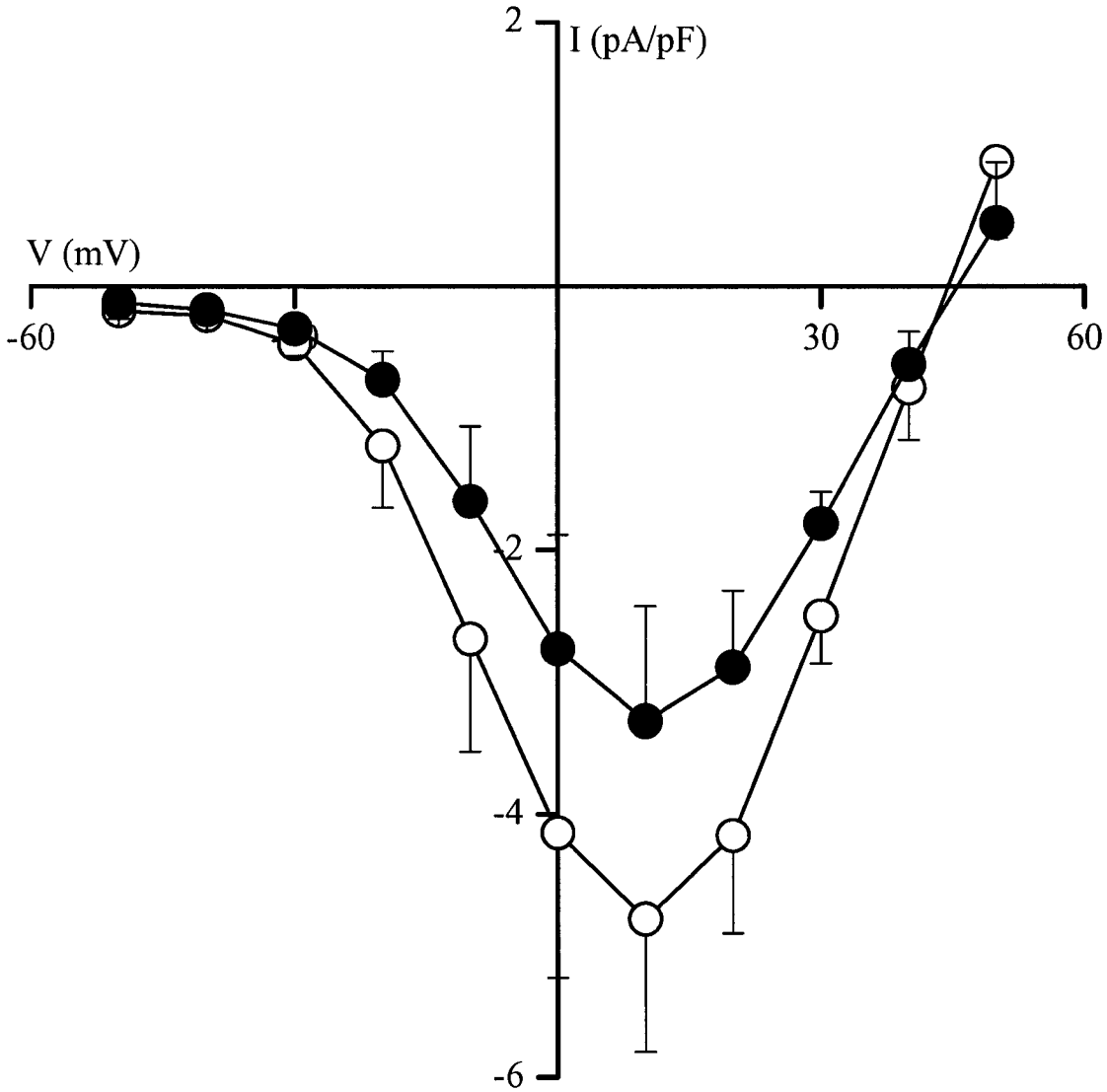
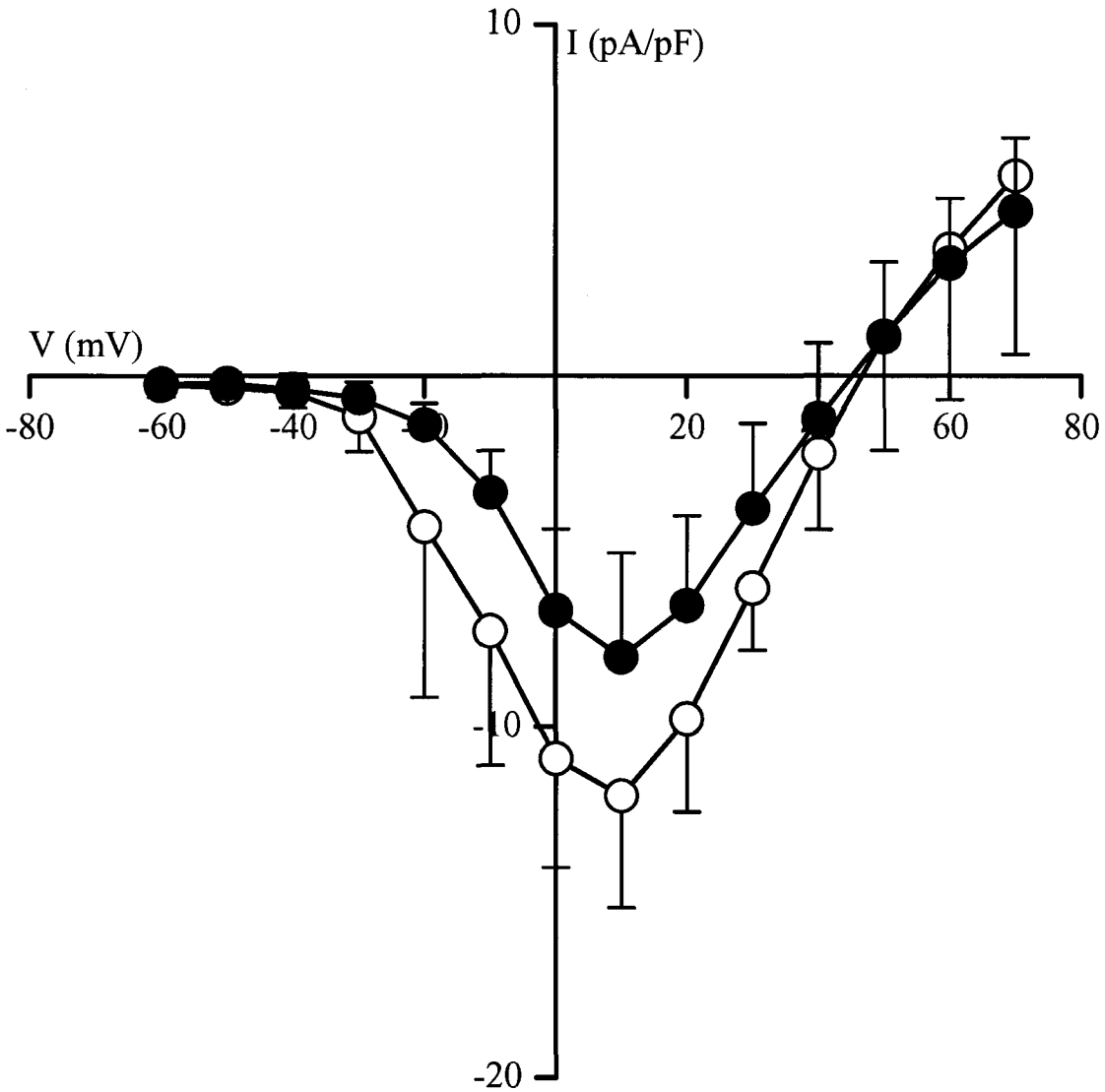

2
I (pA/pF)
V (mV)
-60
30
60
-2
-4
-6

Figure 9: Incubation with catalase inhibited L-type Ca^{2+} channel currents. Mean (± S.E.M) current density-voltage relationships obtained from cells incubated in catalase-free media (○, n = 5) and cells incubated in media containing 500 units/ml catalase (●, n = 5). Control cells were also perfused with catalase-free solution during recording while catalase-incubated cells were perfused in extracellular solution containing 500 units/ml catalase. Currents were evoked by step depolarizing cells for 100 ms to indicated test potentials at a frequency of 1 Hz. Current density (I, pA/pF) was obtained by dividing the evoked current by the cell capacitance.

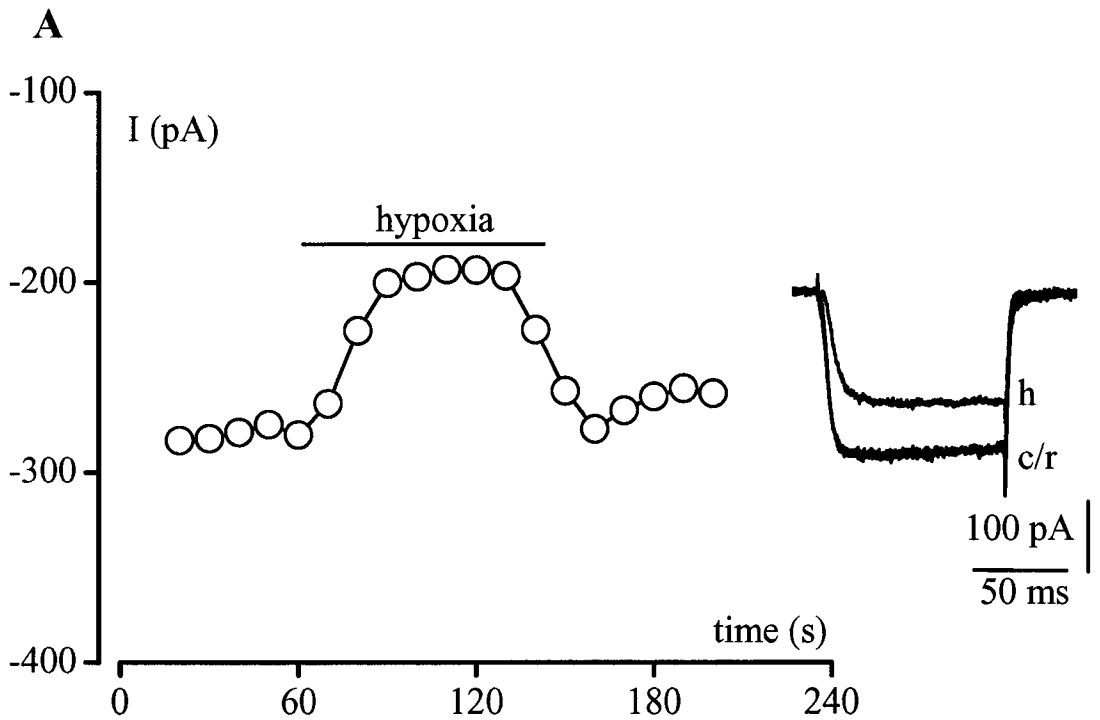

10
I (pA/pF)
V (mV)
-80
-60
-40
20
60
80
-10
-20

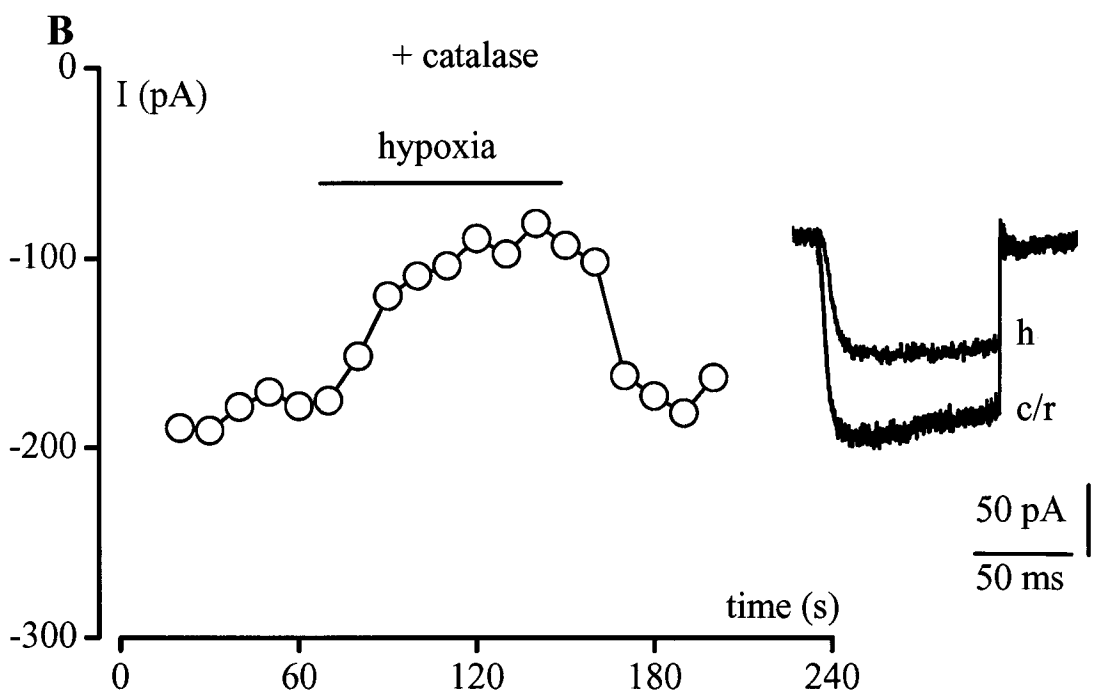
Since H_2O_2 levels modulated Ca^{2+} currents through recombinant L-type Ca^{2+} channels (see above), and H_2O_2 acted as a mediator of hypoxic regulation of K^+ channels in ductus arteriosus smooth muscle cells (Michelakis *et al.*, 2002a), it was of interest to investigate whether H_2O_2 levels mediated the acute hypoxic response of the Ca^{2+} channels. The effects of hypoxia were examined in cells dialyzed without or with 1000 units/ml catalase (Figures 10A and B respectively). In both sets of cells a robust inhibition of Ca^{2+} current was observed during exposure to acute hypoxia. In cells dialyzed with catalase, the magnitude of the O_2-sensitive Ca^{2+} current ($ICaO_2$, calculated by subtracting currents evoked at a test potential of 0 mV during hypoxia from those evoked in normoxia in the same cell) was -1.4 ± 0.2 pA/pF (n = 5), a value not significantly different from that seen in cells dialyzed with catalase-free intracellular solution (-1.6 ± 0.5 pA/pF, n = 5; $P > 0.05$, unpaired Student's *t*-test). In summary, these results indicate that although H_2O_2 levels regulated Ca^{2+} channel activity, alteration of these levels did not mediate the acute hypoxic response of the Ca^{2+} channel.

Superoxide dismutase abrogated acute hypoxic inhibition of L-type Ca^{2+} channel α_{1C} subunits

Increased intracellular H_2O_2 levels were previously shown to modulate the activity of L-type Ca^{2+} channel subunits. In addition to alteration of H_2O_2 levels, altered levels of other forms of ROS have also been shown to modify ion channel activity.

Figure 10: Altered levels of H_2O_2 did not modulate acute hypoxic inhibition of the α_{1C} subunit of the L-type Ca^{2+} channel. Example time-series recordings show the effect of hypoxia (horizontal bars; $Po_2 \sim 20$ mmHg) in; (A) cells exposed to normal intracellular solution (n = 5) and (B) cells dialyzed with 1000 units/ml catalase for 15 min (n = 5). *Inset* show individual current records obtained from the corresponding time-series recording during normoxia (c), during hypoxia (h) and following return to normoxia (r).


A
-100
I (pA)
hypoxia
-200
-300
-400
h
c/r
100 pA
50 ms
time (s)
0
60
120
180
240


B
0
+ catalase
I (pA)
hypoxia
-100
-200
-300
h
c/r
50 pA
50 ms
time (s)
0
60
120
180
240

Previous studies have shown that ATP-sensitive K^+ channel currents increase in response to increases in intracellular O_2^- production (Tokube *et al.*, 1998). Furthermore, L-type Ca^{2+} channels are inhibited 2,5,-Di-t-butyl-1,4-benzohydroquinone (BHQ), which is an inhibitor of the sarco-endoplasmic recticulum Ca^{2+}-ATPase (SERCA) (Fusi *et al.*, 2001). This inhibition of channel function was caused by the increase in formation of O_2^-, as addition of superoxide dismutase (SOD) reversed the inhibitory effect. It has also been demonstrated that the hypoxic pulmonary vasoconstriction response can be attenuated by the addition of an O_2^- scavenger, nitro blue tetrazolium, which prevents H_2O_2 formation (Weissmann *et al.*, 1998). Thus, the aim of this study was to investigate the response of α_{1C} subunits when endogenous O_2^- levels were decreased and H_2O_2 levels were increased by cell dialysis with SOD, which catalyzes the reaction that dismutates superoxide radicals (O_2^-) into H_2O_2. 250 units/ml SOD was added to the intracellular diffusate and 15 min was allowed for the enzyme to dialyze into the cells. Figure 11 shows that addition of SOD inhibited Ca^{2+} currents under normoxic conditions, for example such that at a test potential of +10 mV, currents were -6.56 ± 1.59 pA/pF (control; $n = 7$) and -3.12 ± 0.93 pA/pF in cells dialyzed with SOD ($n = 5$; $P > 0.05$, unpaired Student's *t*-test). Despite this inhibition of basal Ca^{2+} currents, SOD was without effect on the response to acute hypoxia, since in the presence of SOD hypoxia caused no further decrease in Ca^{2+} current amplitudes (Figure 12). For example in SOD dialyzed cells currents evoked at a test potential of +10 mV were -3.2 ± 0.9 ($n = 4$) pA/pF in normoxia and -2.2 ± 0.24 pA/pF ($n = 4$) when cells were subjected to acute hypoxia ($P > 0.05$, paired Student's *t*-test). Figure 13 shows the mean current amplitudes evoked at +10 mV

Figure 11: Superoxide dismutase (SOD) inhibited Ca^{2+} currents through recombinant L-type Ca^{2+} channel α_{1C} subunits. Mean (± S.E.M) current density-voltage relationships obtained from cells dialyzed with SOD-free intracellular solution (○, n = 7) or cells dialyzed with 250 units/ml SOD (●, n = 5) for 15 min prior to recording. Currents were evoked by step depolarizing cells for 100 ms to indicated test potentials at a frequency of 1 Hz. Current density (I, pA/pF) was obtained by dividing the evoked current by the cell capacitance.

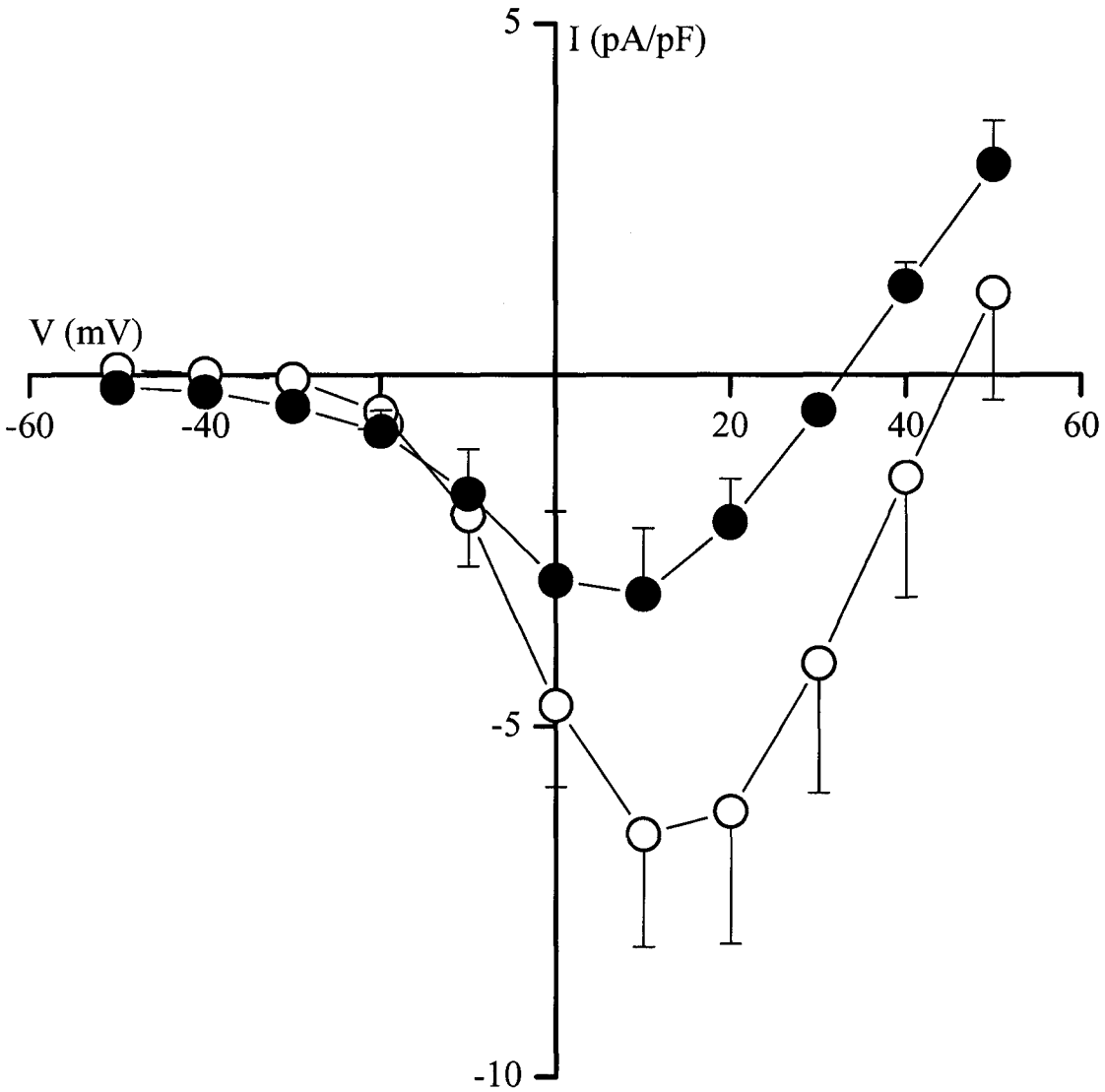

5
I (pA/pF)
V (mV)
-60
-40
20
40
60
-5
-10

Figure 12: SOD abrogated hypoxic inhibition of the α_{1C} subunit of L-type Ca^{2+} channels. Mean (± S.E.M) current density-voltage relationships obtained from cells dialyzed with 250 units/ml SOD for 15 min prior to recording (○, n = 4) and SOD dialyzed cells subjected to acute hypoxia (●, n = 4) (Po_2~ 20 mmHg). Currents were evoked by step depolarizing cells for 100 ms to indicated test potentials at a frequency of 1 Hz. Current density (I, pA/pF) was obtained by dividing the evoked current by the cell capacitance.

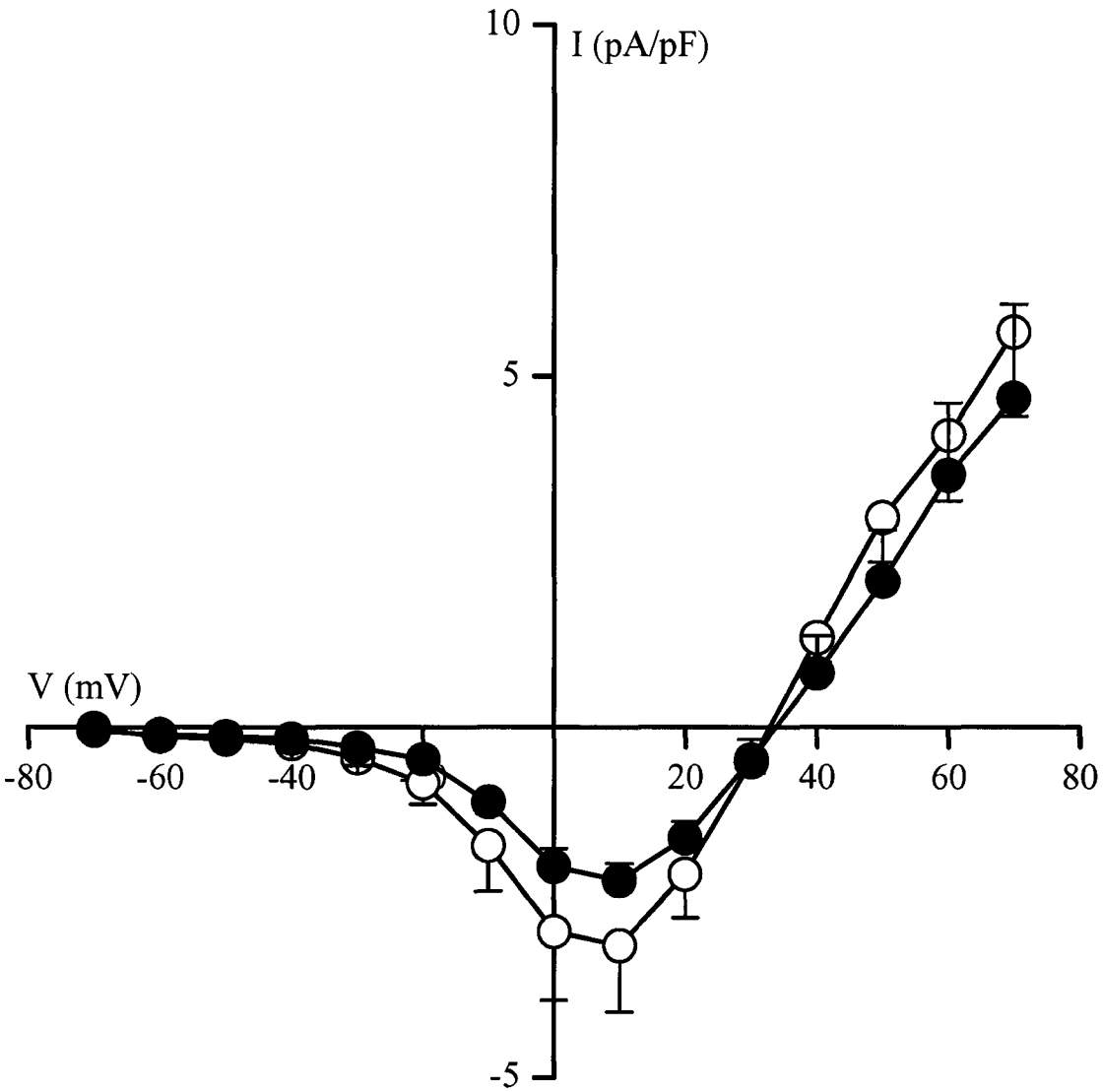
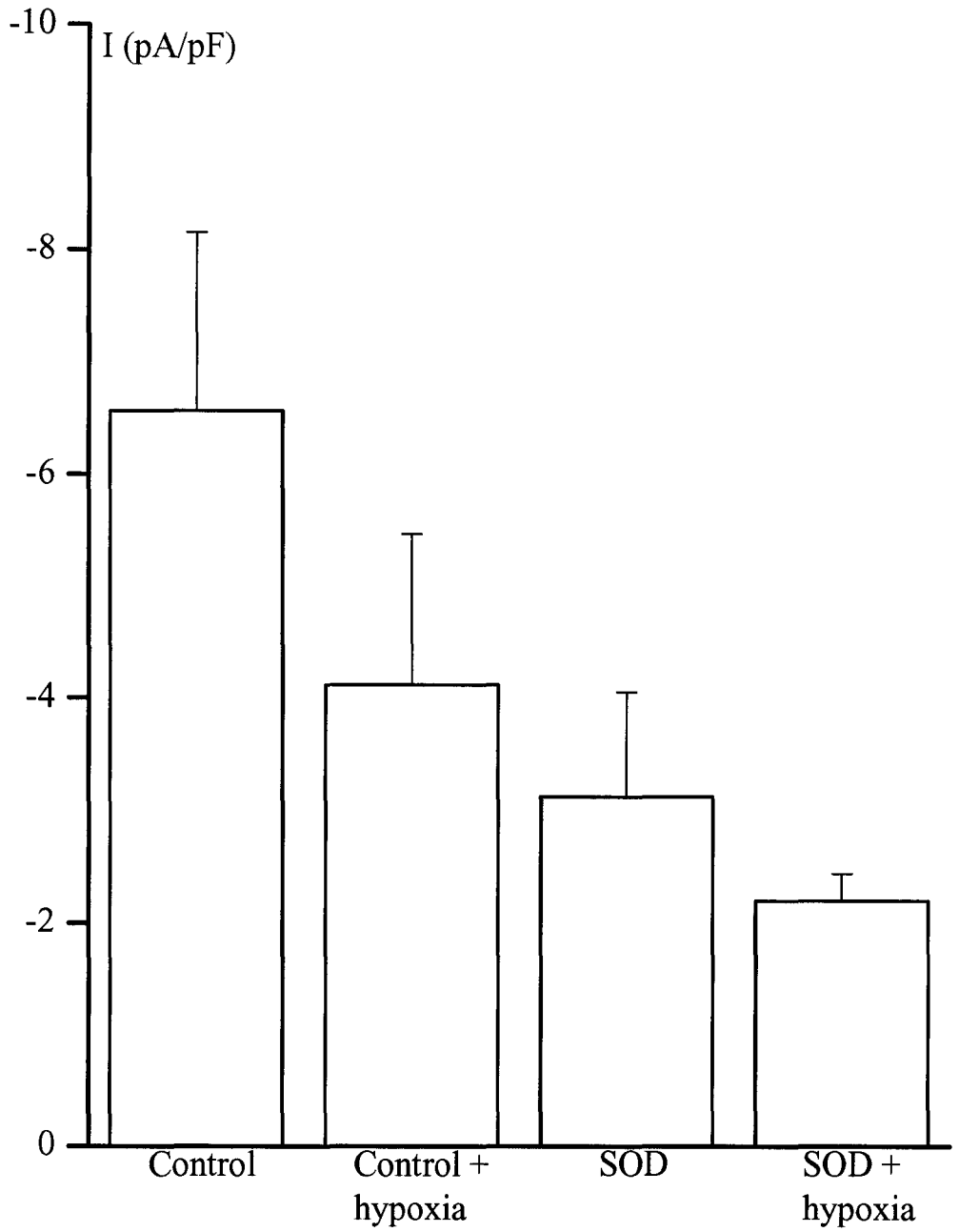

10
I (pA/pF)
5
V (mV)
-80
-60
-40
20
40
60
80
-5

Figure 13: SOD mimicked the effects of hypoxia in HEK 293 cells expressing recombinant L-type Ca^{2+} channels α_{1C} subunits. Mean (± S.E.M) magnitudes of current density were obtained from control cells (n = 7), control cells subjected to acute hypoxia (n = 5, Po_2 ~ 20 mmHg), SOD dialyzed cells under normoxic conditions (n = 4) or cells dialyzed with SOD dialyzed cells subjected to acute hypoxia (n = 4, Po_2 ~ 20 mmHg) as indicated. Magnitude of currents were calculated by determining the difference between mean current evoked under normoxic conditions and mean current evoked during acute hypoxia. All values were obtained at a test potential where currents were maximal (+10 mV). Currents were evoked by step depolarizing cells for 100 ms to indicated test potentials at a frequency of 1 Hz. Current density (I, pA/pF) was obtained by dividing the evoked current by the cell capacitance.


-10
I (pA/pF)
-8
-6
-4
-2
0
Control
Control + hypoxia
SOD
SOD + hypoxia

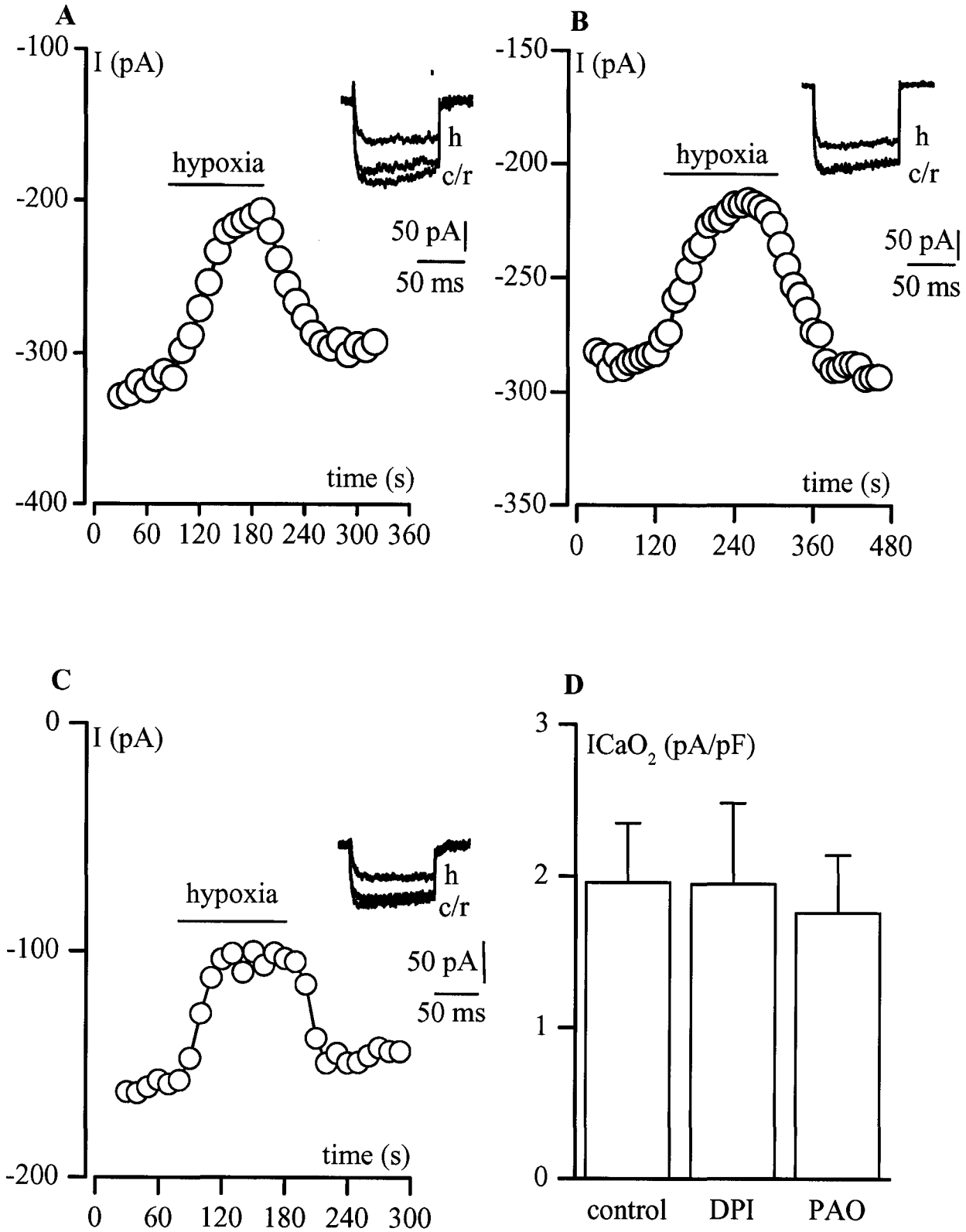
in the experiments in which SOD was utilized. These results demonstrate that SOD regulated Ca^{2+} current and inhibited the acute hypoxic response of recombinant L-type Ca^{2+} channel α_{1C} subunits.

NADPH oxidase did not modulate the acute hypoxic response of α_{1C} subunits

I further examined the involvement of NADPH oxidase as a possible mediator of the acute hypoxic response of recombinant L-type Ca^{2+} channels. NADPH oxidase has been demonstrated to play a role in O_2 sensing in pulmonary neuroepithelial bodies of mice since the absence of a functional NADPH oxidase abolished the hypoxic inhibition of K^+ current (Fu *et al.*, 2000). Using two distinct NADPH oxidase inhibitors, diphenylene iodonium (DPI) and phenylarsine oxide (PAO), the role of NADPH oxidase in the acute hypoxic response of recombinant L-type Ca^{2+} channels was examined.

Figures 14A-C show the acute hypoxic responses of recombinant L-type Ca^{2+} channels in control cells (A), cells exposed to 10 μM DPI (B) and cells exposed to 5 μM PAO (C). Bath application of these drugs did not inhibit the acute hypoxic response of this channel, and the mean data (Figure 14D) demonstrate the similarity of the magnitude of the O_2 sensitive Ca^{2+} current ($ICaO_2$) in the absence and presence of the NADPH oxidase inhibitors. These results indicate that regulation of NADPH oxidase does not mediate the hypoxic response of these recombinant channels.

Figure 14: NADPH oxidase does not modulate the acute hypoxic response of the α_{1C} subunit. **A, B and C**, example time-series recordings demonstrating the inhibitory effect of acute hypoxia (Po_2~ 10 mmHg) on Ca^{2+} channel currents in HEK 293 cells stably expressing human cardiac L-type Ca^{2+} channel α_{1C} subunits. The effects of hypoxia were examined (**A**) under control conditions, (**B**) during exposure to the NADPH oxidase inhibitor diphenylene iodonium (DPI, 10 μM; typical of 5 such recordings), and (**C**) during exposure to a further inhibitor of the oxidase, phenylarsine oxide (PAO, 5 μM; typical of 5 such recordings). Both DPI and PAO were without effect on the ability of hypoxia to inhibit Ca^{2+} channel activity. Currents were evoked by step depolarizing cells to 0 mV from a holding potential of -80 mV for 100 ms every 10 s. Inset shows individual current records obtained from the corresponding time-series recording in normoxia (c), during hypoxia (h) and following return to normoxia (r). **C**, magnitude of $ICaO_2$ (as calculated by determining the difference between mean current evoked during normoxia and mean current evoked during acute hypoxia) under control conditions and in the presence of DPI and PAO. Data were plotted as means (± S.E.M) from 5 recordings for both DPI and PAO. No significant differences were seen between control and test groups.


A
-100
I (pA)
hypoxia
h
c/r
50 pA
50 ms
-200
-300
-400
time (s)
0 60 120 180 240 300 360
B
-150
I (pA)
hypoxia
h
c/r
50 pA
50 ms
-200
-250
-300
-350
time (s)
0 120 240 360 480
C
0
I (pA)
hypoxia
h
c/r
50 pA
50 ms
-100
-200
time (s)
0 60 120 180 240 300
D
3
ICaO2 (pA/pF)
2
1
0
control
DPI
PAO

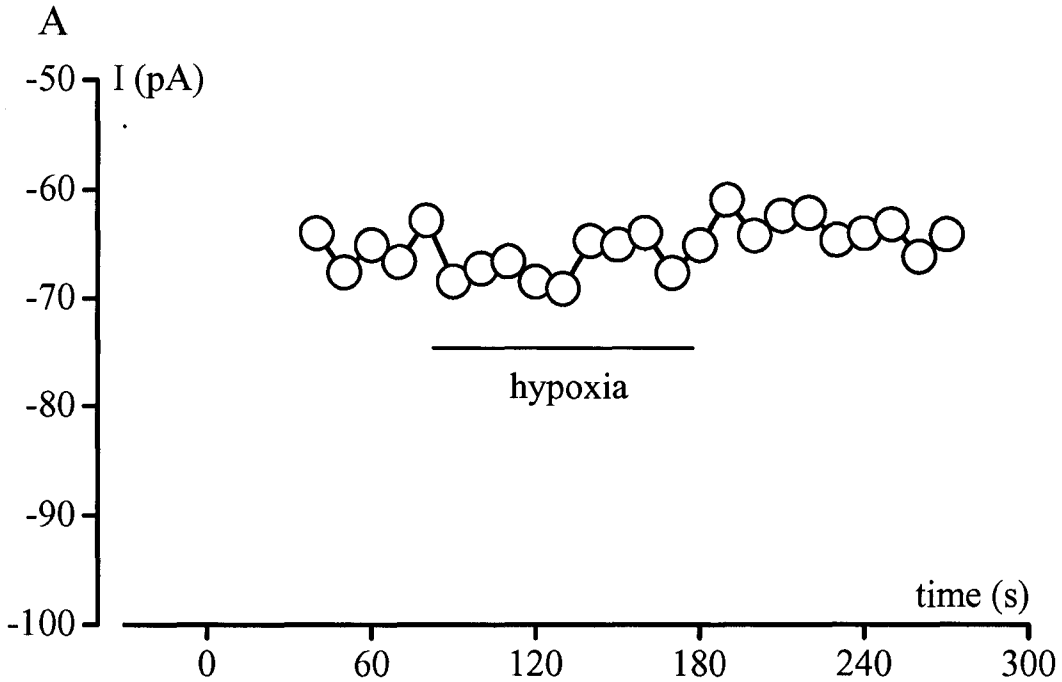
Functional mitochondria are required for acute hypoxic sensing by the α_{1C} subunit

The aim of this study was to investigate the role mitochondria play in O_2 sensing by recombinant L-type Ca^{2+} channel α_{1C} subunits. Mitochondria and their electron transport chain complexes have been proposed as mediators of O_2 sensing (Archer *et al.*, 1993; Waypa *et al.*, 2001; Lopez-Barneo *et al.*, 2004). A cell line was established in which mitochondrial DNA was selectively depleted via long-term treatment with ethidium bromide at a concentration sufficient to deplete mitochondrial DNA (mtDNA) (Searle *et al.*, 2002). These ρ^0 cells were supplemented with uridine and pyruvate to provide an alternate source of ATP. Figures 15A and B show time-series recordings of Ca^{2+} currents obtained in wild-type (ρ^+) and ρ^0 cells. When recording from ρ^0 cells, acute hypoxia caused no change in Ca^{2+} current amplitudes (e.g. Figure 15A), which is in stark contrast to the hypoxic inhibition of Ca^{2+} currents in ρ^+ cells (Figure 15B). The mean (± S.E.M) magnitude of the O_2 sensitive current ($ICaO_2$) was 2.42 ±1.5 pA/pF ($n = 11$) in ρ^+cells and 0.17 ± 0.29 pA/pF ($n = 20$) in ρ^0 cells ($P < 0.05$, unpaired Student's *t*-test; Figure 16). These results suggest that functional mitochondria are required for O_2 sensing by recombinant L-type Ca^{2+} channel α_{1C} subunits.

DISCUSSION

Ion channels in excitable cells function to regulate responses in a variety of extracellular and intracellular signals. In addition, ion channels themselves can be

Figure 15: Acute O_2 sensing was abolished in the absence of a functional mitochondrial electron transport chain. (A) Example time-series recording made in a ρ^0 cell which was exposed to acute hypoxia (Po_2~ 20 mmHg) for the period indicated by the horizontal bar (typical of 20 such recordings). (B) Example time-series recording of acute hypoxic responses in control cells (Po_2~ 20 mmHg, horizontal bar, typical of 11 such recordings). In both panels, currents were evoked by step depolarizing the cells to 0 mV from a holding potential of -80 mV for 100 ms at a frequency of 0.1 Hz.


A
-50
I (pA)
-60
-70
-80
-90
-100
hypoxia
time (s)
0
60
120
180
240
300

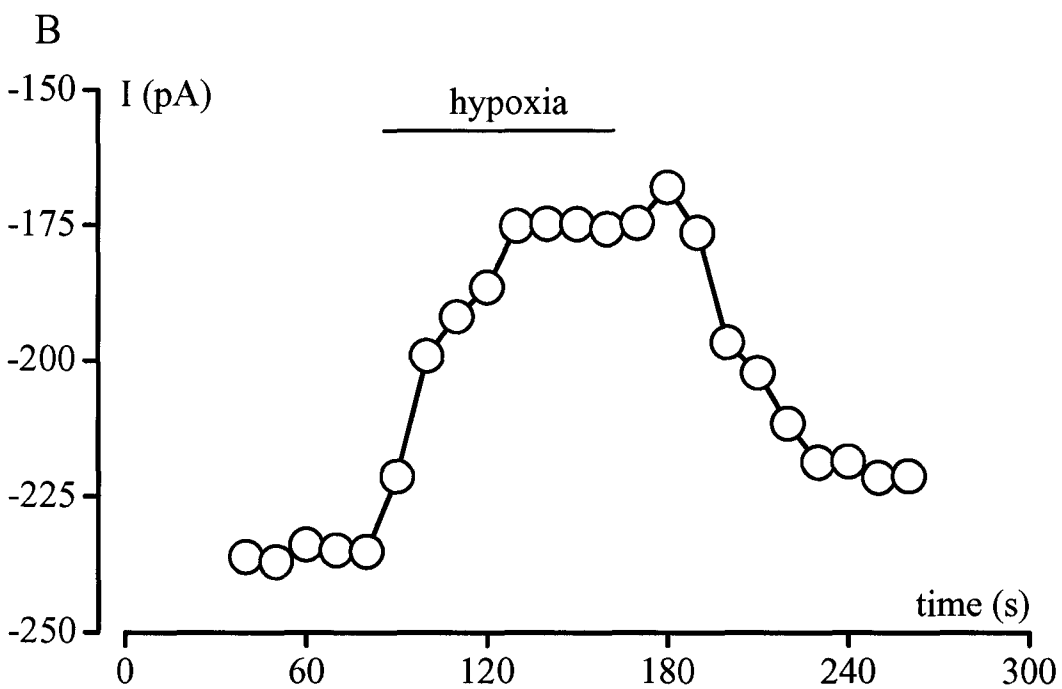
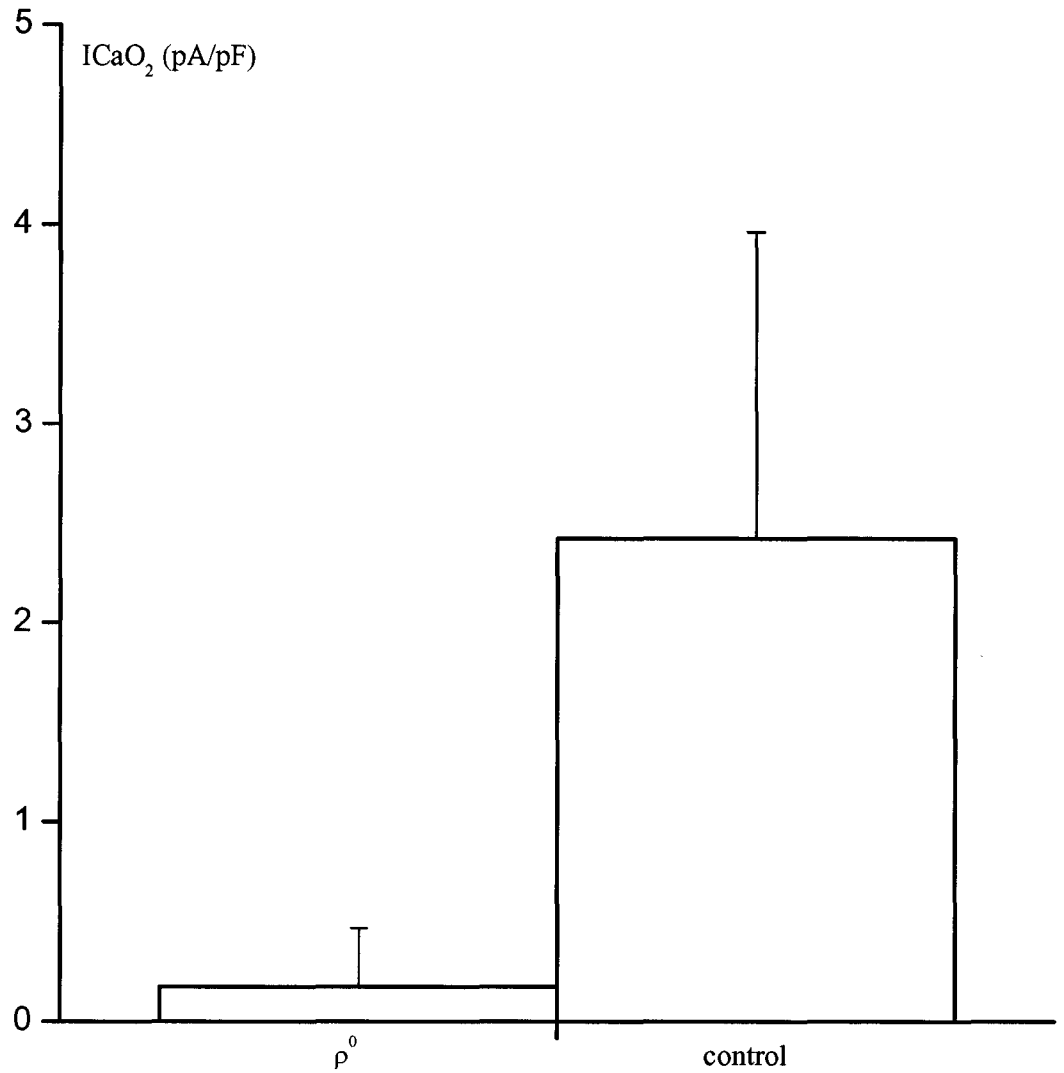

B
-150
I (pA)
hypoxia
-175
-200
-225
-250
time (s)
0
60
120
180
240
300

Figure 16: Mean (± S.E.M) magnitude of the O_2-sensitive Ca^{2+} current ($ICaO_2$) was obtained from time-series recordings in ρ^0 cells (n = 20) and ρ^+ cells (n = 11) by subtracting current amplitudes evoked by hypoxia from those obtained during normoxia in the same cell. Results were found to be significant ($P < 0.05$, Student's unpaired *t*-test).


5
4
3
2
1
0
ICaO2 (pA/pF)
ρ0
control

regulated by numerous extracellular and intracellular signals. Acute hypoxia is one such extracellular signal that is known to regulate specific types of ion channels. During acute hypoxia, regulation of ion channels initiates a host of physiological responses which are cell-type specific. For example, in carotid body type I cells, hypoxia inhibition of K^+ channels ultimately leads to neurotransmitter release (Pardal *et al.*, 2000) while in pulmonary vascular smooth muscle cells, the inhibition of the same channels may lead to vasoconstriction (Post *et al.*, 1992). The aim of these physiological responses is to ensure oxygenated blood is delivered to tissues at levels suitable to metabolic need. In systemic vascular smooth muscle cells, exposure to acute hypoxia results in relaxation of these cells, which leads to a vasodilatory response (Marriott and Marshall, 1990; Vadula *et al.*, 1993), an effect at least partly mediated by L-type Ca^{2+} channels found in these cells.

HEK 293 cells do not normally express L-type Ca^{2+} channels but do express several types of background K^+ channels (Shaw *et al.*, 2002). Recombinant expression of L-type Ca^{2+} channel can be accomplished by stable transfection of wild-type HEK 293 cells with cDNA copies of the α_{1C} subunit. All experiments described in this thesis utilized HEK 293 cells stably expressing the pore forming α_{1C} subunit of the L-type Ca^{2+} channel (Schultz *et al.*, 1993). Cells were shown to stably express functional subunits since the application of nifedipine, an L-type Ca^{2+} channel blocker, effectively inhibited all inward Ca^{2+} currents.

L-type Ca^{2+} channels were first shown to be O_2 sensitive in isolated rabbit vascular myocytes (Franco-Obregon *et al.*, 1996a). Similar to O_2-sensitive K^+ channels, L-type Ca^{2+} channels responded to acute hypoxia by decreasing the amount of current flow through them in response to stimulation. Later studies demonstrated that acute hypoxia directly inhibited L-type Ca^{2+} channels in rat vascular smooth muscle cells and human coronary myocytes (Herrera and Walker, 1998; Smani *et al.*, 2002). Further studies demonstrated that L-type Ca^{2+} channels, expressed in a recombinant system, are inhibited during acute hypoxia and more specifically it is the α_{1C} subunits of these channels that are inhibited by acute hypoxia (Fearon *et al.*, 1997 and 1999). Hypoxic responses in this system were voltage-dependent and Ca^{2+} currents were inhibited in a manner similar to that seen in native L-type Ca^{2+} channels. My data were in accordance with these previously published report as acute hypoxia was found to reversibly inhibit Ca^{2+} current in HEK 293 cells stably expressing L-type Ca^{2+} channel α_{1C} subunits in a voltage-dependent manner.

The exact mechanism of how this channel is inhibited by hypoxia is not fully understood, but one theory proposes that redox modulation of cysteine residues in this subunit modifies channel activity so as to inhibit Ca^{2+} current in response to acute hypoxia (Fearon *et al.*, 1999). Redox modulation could occur by altering production or levels of reactive O_2 species (ROS) in relation to the amount of available O_2. Less ROS would be generated by NADPH oxidase, or by the mitochondrial electron transport chain

as less O_2 becomes available, leading to alterations in ratios of regulatory redox couples such as glutathione (GSSG/GSH) and NAD (P)$^+$/NAD (P) H (Peers, 1997).

The reactive O_2 species H_2O_2 is a proposed mediator of hypoxic signaling in pulmonary neuroepithelial bodies (Wang *et al.*, 1996), their immortalized counterparts H146 cells (O'Kelly *et al.*, 2000) and cultured pulmonary arterial myocytes (Waypa *et al.*, 2002). Recently H_2O_2 was shown to modulate K^+ channel currents in HUVEC cells in a dose-dependent manner (Bychkov *et al.*, 1999). In this study, higher concentrations of H_2O_2 (1 mmol) were shown to increase amplitude of outwardly rectifying K^+ channel while lower concentration of H_2O_2 (0.01 to 0.25 μmol) inhibited inward-rectifying K^+ current. Given the proposed role for H_2O_2 in O_2 sensing, I examine the effects of H_2O_2 and a variant, tert-butyl hydroperoxide, and showed that exogenous addition of these compounds to cells expressing α_{1C} subunits caused significant enhancement of Ca^{2+} currents. The effects of H_2O_2 on current enhancement were voltage-dependent, which was similar to the effect of acute hypoxia on native Ca^{2+} channels (Franco-Obregon *et al.*, 1996b) and recombinant α_{1C} subunits (Fearon *et al.*, 1997). These data are also supported by recent findings that H_2O_2 enhances Ca^{2+} current through L-type Ca^{2+} channels in cultured rat dentate granule cells although H_2O_2 did not affect voltage-dependence (Akaishi *et al.*, 2004). A possible reason for the lack of voltage-dependence in this system was that Akaishi's group used a much lower concentration of H_2O_2 (1 μM) and also incubated cells in H_2O_2 for 2 h prior to electrophysiological recordings. Cells did not survive when higher concentrations of H_2O_2 (20-200 μM), similar to those

utilized in this thesis, were used. As such, it is possible that the voltage-dependence seen in this thesis occurred at higher but not lower concentrations of H_2O_2.

To examine the role of endogenous H_2O_2 in mediating recombinant Ca^{2+} channel currents, catalase was added to the intracellular solution and was allowed to dialyze into the cell (via the patch pipette) for sufficient time to reach equilibrium (Pusch and Neher, 1988). Dialysis with catalase resulted in decreased Ca^{2+} currents compared to cells that were dialyzed with catalase-free intracellular solution. Likewise in cells incubated with catalase for 24 h prior to recording, Ca^{2+} currents were inhibited compared to Ca^{2+} currents in cells incubated for 24 h in the absence of catalase. These results were consistent with my observations that H_2O_2 activated Ca^{2+} channels, such that a decrease in Ca^{2+} current amplitudes is related to the decrease in the amount of endogenous H_2O_2 due to its breakdown by catalase. It is also consistent with a previous report that showed H_2O_2-induced increase in intracellular Ca^{2+} could be attenuated in rat pulmonary arterial myocytes by over-expressing catalase by adenovirus transfection (Waypa *et al.,* 2002). Like H_2O_2, the effects of catalase on Ca^{2+} currents were moderately voltage-dependent, with the greatest degree of inhibition occurring at test potentials where currents were maximal.

These data are in accordance with the hypothesis that H_2O_2, normally produced during normoxia, is reduced from basal levels during hypoxia such that Ca^{2+} current activity is inhibited. This hypothesis stems from previous studies that found H_2O_2 levels

decreased during hypoxia in H146 cells (O'Kelly *et al.*, 2000) and cardiac myocytes (Hool, 2000). In the H146 cells, K^+ currents and cellular H_2O_2 levels were found to be suppressed in hypoxic conditions and exogenous H_2O_2 application was found to reactivate K^+ currents (O'Kelly *et al.*, 2000). Additionally, studies using human ductus arteriosus, an O_2 sensitive blood vessel in the heart that allows venous blood flow to bypass the lungs in a fetus, found that complex red-reactive H_2O_2 levels decreased during hypoxia as did K^+ currents (Michelakis *et al.*, 2002a). Studies in bovine pulmonary arteries also show that intracellular H_2O_2 levels were mediated by O_2 tension, such that hypoxia decreased the levels of this ROS (Burke-Wolin and Wolin, 1989 and 1990). These data support a strong link between hypoxia, H_2O_2 levels and ion channel inhibition. To test this hypothesis, I examined hypoxic responses of recombinant L-type Ca^{2+} channel currents in cells where H_2O_2 levels were ablated by catalase. The concentration of catalase used in these studies (1000 units/ml) was higher than concentrations previously shown to regulate the sensitivity of the L-type Ca^{2+} channel to isoproterenol (Hool and Arthur, 2002), and as I indicated previously exerted an inhibitory effect on Ca^{2+} channel currents. Acute hypoxia still caused robust inhibition in cells that were dialyzed with catalase for 15 min prior to time-series recording, a response much like the inhibitory response seen in cells dialyzed with catalase-free intracellular solution and subsequently exposed to acute hypoxia. The magnitude of O_2 sensitive current was not significantly different in cells dialyzed in the absence or presence of catalase. Taken together, these data demonstrate that while exogenous H_2O_2 and ablation of cellular H_2O_2

levels activate and inhibit the Ca^{2+} channels respectively, altered production of this ROS does not mediate hypoxic inhibition of recombinant L-type Ca^{2+} channels.

It was possible that although alteration of H_2O_2 levels by catalase did not have an effect on the hypoxic response of recombinant L-type Ca^{2+} channels, alternative forms of ROS, such as O_2^-, could mediate hypoxic sensitivity. In accordance with this hypothesis, cell dialysis with superoxide dismutase (SOD), an enzyme responsible for the dismutation of O_2^- into H_2O_2, both mimicked and abrogated the inhibitory response to acute hypoxia. These data are in agreement with previous findings that showed addition of SOD in the presence of an O_2^- generator, Sin-1 inhibited L-type Ca^{2+} currents in ferret ventricular myocytes (Campbell *et al.*, 1996). In addition, SOD inhibited Kv1.5 K^+ channels expressed in CHO cells (Caouette *et al.*, 2003). Furthermore, in pulmonary arterial smooth muscle cells, SOD abolished hypoxia-induced constriction (Lui *et al.*, 2003), which is dependent on K^+ current regulation (Weir and Archer, 1995). In addition the SOD mimetic tempol attenuated the hypoxic response of rat ventricular myocytes such that the development of pulmonary hypertension, normally caused by chronic hypoxia, was inhibited (Elmedal *et al.*, 2004). Under chronically hypoxic conditions, O_2^- and other forms of ROS were shown to contribute to the chronic hypoxic response of ventricular myocytes, which resulted in pulmonary hypertension and hypertrophy of cells. When tempol was added to this system, the chronically hypoxic response of these cells was blunted. Although the mechanism was not fully understood, it is clear from these and my own studies that SOD and forms of this enzyme might play a role in

mediating not only the chronic hypoxic response but possibly the acute hypoxic response of recombinant Ca^{2+} channels. This thesis is the first report to demonstrate the involvement of O_2^- production in acute hypoxic inhibition of recombinant L-type Ca^{2+} channels. These findings necessitate further studies using xanthine-xanthine oxidase (which produces O_2^-) to further examine the role of this ROS in L-type Ca^{2+} channel O_2 sensing.

A further proposed mediator of acute hypoxic signaling is the multi-subunit enzyme NADPH oxidase, which catalyzes the reaction that produces O_2^- from O_2, which is further dismutated to H_2O_2 by SOD. NADPH oxidase has been proposed to mediate altered ion channel activity in response to acute hypoxia in model and isolated lung neuroepithelial cells (O'Kelly *et al.*, 2000; Fu *et al.*, 2000) and carotid body type I cells (Cross *et al.*, 1990). HEK 293 cells express a catalytic isoform of the gp91^{phox} subunit of NADPH oxidase, NOX4, which is the main subunit that catalyzes the reaction that produces O_2^- from molecular O_2 and is also sensitive to the NADPH oxidase inhibitor by diphenylene iodonium (DPI) (Shiose *et al.*, 2001). No evidence was found in my results that showed a role for NADPH oxidase in hypoxic inhibition of recombinant L-type Ca^{2+} channel α_{1C} subunits. DPI and phenylarsine oxide (PAO), another NADPH oxidase inhibitor that binds to the active site of NADPH oxidase (Doussiere *et al.*, 1998), were used at concentrations in excess of those that have previously been shown to modulate ion channel function and suppress the effects of acute hypoxia (O'Kelly *et al.*, 2000). Even at high concentrations, no effect of these drugs was observed on the acute hypoxic

inhibition of the recombinant Ca^{2+} channels. This suggests that NADPH oxidase does not regulate Ca^{2+} channel activity under hypoxic conditions. Interestingly, DPI has been proposed to directly block Ca^{2+} current in rat carotid body type I cells (Wyatt *et al.*, 1994). L-type Ca^{2+} channels are the most significant type of channel that carries Ca^{2+} current in the rat carotid body (Stea *et al.*, 1995; Peers *et al.*, 1996). When taking this into consideration with the present data concerning the lack of effect of DPI or PAO on acute hypoxic inhibition of α_{1C} subunits of L-type Ca^{2+} channels, it suggests that while L-type Ca^{2+} channels in rat carotid body are affected by DPI, the hypoxic response of recombinant channels in HEK 293 cells is not affected by the presence of DPI.

The above results have shown that NADPH oxidase does not mediate recombinant L-type Ca^{2+} channel responses to acute hypoxia thus, the next point of interest comes from an alternative model of O_2 sensing which involves mitochondria. Mitochondria have been proposed to play a role in O_2 sensing due to the fact that they utilize a vast majority of cellular O_2 in order to carry out oxidative phosphorylation to generate ATP. In doing so they generate the vast majority of ROS in cells due to inefficient electron transport through the ETC. It has previously been shown that parts of the mitochondria can modulate the hypoxic response of ion channels. Cytochrome a3 of the mitochondrial ETC in cat carotid body cells has been shown to be the primary O_2 sensor (Wilson *et al.*, 1994). Mitochondria have also been shown to account for the diversity in O_2 sensing in vascular cells of pulmonary and renal arteries, which constrict and relax, respectively, in response to hypoxia (Michelakis *et al.*, 2002b). This study

reported that the difference in mitochondrial derived ROS between pulmonary arterial cells and renal arterial cells, of which the former produces more, might account for the differing responses to hypoxia. The authors showed that inhibitors of the mitochondrial ETC alter K^+ channel activity in both cell types in a similar manner to that seen under acute hypoxia. Even more recently, it has been shown that Complex I and III of the mitochondrial ETC of rat carotid body type I cells are required for O_2 sensing. Addition of mitochondrial inhibitors like rotenone and cyanide abolished hypoxic inhibition of background K^+ channels (Wyatt and Buckler, 2004). Further studies have been conducted with the use of cells lacking functional mitochondrial DNA (ρ^0) cells. These cells provide great insight into cellular mechanisms required by redox regulation changes or mitochondrial ROS generation. It was previously found that ρ^0 Hep3B cells did not show an increase in 2, 7-dichlorofluorescein (DCFH) oxidation when these cells were subjected to acute hypoxia (Chandel *et al.*, 1998). DCFH oxidation suggests an increase in ROS generation, a response that occurs when wild-type Hep3B cells were exposed to acute hypoxia. From this it was concluded that functional mitochondria were required for the hypoxic response in Hep3B cells. In ρ^0 HEK 293 cells, acute hypoxic inhibition of Ca^{2+} current through L-type Ca^{2+} channel α_{1C} subunits was significantly reduced compared to wild-type (ρ^+) HEK 293 cells that possessed functional mitochondria. The magnitude of O_2 sensitive Ca^{2+} current was also significantly reduced in ρ^0 cells such that it was hard to distinguish the difference between current amplitudes prior to and during application of acute hypoxia. Other studies using ρ^0 cells to study the role of mitochondrial in O_2 sensing have shown that functional mitochondria are necessary for

the transcriptional response to hypoxia in Hep3B cells and HEK 293 cells (Chandel *et al.*, 1998; Chandel *et al.*, 2000). This transcriptional response is characterized by an increase in HIF-1 protein and erythropoietin mRNA in response to prolonged periods of hypoxia (Chandel *et al.*, 1998). Although the amount of time cells spent in hypoxic conditions was significantly greater than in my experiment, both results from Chandel's group demonstrated that functional mitochondria play a significant role in O_2 sensing. These data also support the model that mitochondrial generation of ROS is important in O_2 sensing. Without functional mitochondrial DNA, oxidative phosphorylation would not occur, loss of electrons from mitochondrial complexes would not take place and less ROS would be generated to act as second messengers in the O_2 sensing pathway.

In summary, nifedipine was found to inhibit inward Ca^{2+} current in L-type Ca^{2+} channel α_{1C} subunits, an expected result, as nifedipine is an L-type Ca^{2+} channel blocker. Acute hypoxia was also found to inhibit Ca^{2+} currents through these recombinant channels in a reversible and voltage-dependent manner. Ca^{2+} channel activity was regulated by basal levels of H_2O_2 through interaction with the pore-forming α_{1C} subunit. Neither alteration of H_2O_2 levels by catalase nor regulation of the H_2O_2-contributor NADPH oxidase were involved in the acute hypoxic regulation of this channel. Alteration in O_2^- levels by the presence of SOD did modify the inhibitory hypoxic response of recombinant channels, as did the absence of functional mitochondrial DNA. The latter is perhaps the most significant observation since mitochondria, which produce O_2^-, and SOD, which alters the levels of O_2^-, were both found to alter the hypoxic

response of recombinant L-type Ca^{2+} channels. Taken together, these data support a model in which altered production of O_2^- within the mitochondrial ETC during hypoxia mediates acute O_2 sensing by the Ca^{2+} channel. Further experiments examining alterations in the hypoxic response of these recombinant channels due to altered O_2^- levels will need to be conducted in order to validate my hypothesis.

CHAPTER 2

Investigating the role of ROS-producing complexes on the chronically hypoxic response of L-type Ca^{2+} channel α_{1C} subunits

SUMMARY

The chronic hypoxic response of recombinant cells stably expressing L-type Ca^{2+} channels was examined in this study. Factors proposed to modulate channel activity in relation to chronic hypoxia were investigated with the use of pharmacological inhibitors. All experiments were conducted in HEK 293 cell stably expressing L-type Ca^{2+} channel α_{1C} subunits. Cells were incubated in normoxic or chronically hypoxic conditions, in the absence or presence of drugs, depending on the experiment.

Chronic hypoxia significantly enhanced Ca^{2+} currents through L-type Ca^{2+} channel α_{1C} subunits. Application of rotenone, a mitochondrial ETC complex I inhibitor, had no effect on Ca^{2+} currents during normoxic conditions but abolished the enhancement of Ca^{2+} currents due to chronic hypoxia. In addition to this finding, diphenylene iodonium (DPI), an NADPH oxidase inhibitor, was without effect on Ca^{2+} currents under normoxic conditions or chronically hypoxic conditions. These data indicate that complex I in the mitochondrial ETC, is an integral part in regulation of Ca^{2+} currents through L-type Ca^{2+} channel α_{1C} subunits in response to chronic hypoxia.

INTRODUCTION

Chronic hypoxia (CH) occurs when cells receive inadequate amounts of O_2 for prolonged periods of time. Many diseases exist in human pathology that are in part due to CH such as chronic hypertension, chronic obstructive pulmonary disorder and have been proposed to include dementias such as Alzheimer's disease. During CH, cells undergo a variety of physiological changes which can include disruption of Ca^{2+} homeostasis and increased oxidative stress (Peers, 2002). Altered Ca^{2+} homeostasis can lead to constriction in the pulmonary vasculature, dilation in the systemic vasculature and has been linked to the formation of amyloid β plaques, a hallmark of Alzheimer's disease (Peers, 2002). CH also alters the expression of ion channel genes, which is thought to be guided by increased amounts of hypoxia-inducible factor-1 (HIF-1). The exact mechanism of how CH is able to regulate ion channel activity is still not understood as there is still speculation as to what molecule(s) play a role in O_2 signaling.

Most experiments have investigated the K^+ channel response to CH. In all cases, K^+ channels in tissues like carotid body glomus cells and pulmonary arterial myocytes are inhibited during exposure to CH such that there is a decrease in current density (Smirnov *et al.*, 1994; Wang *et al.*, 1997). It was later demonstrated that the underlying reason for decreases in channel currents was mainly due to a decrease in K^+ channel mRNA such that there was decreased expression of the channel. Ca^{2+} channels have also been demonstrated to be affected by CH. In rat carotid body cells, Ca^{2+} currents were

enhanced after exposure to CH (Peers *et al.*, 1996). Ca^{2+} current enhancement was later thought to be due to an increase in ROS production (Green *et al.*, 2002). Increased ROS levels have been linked to increases in amyloid β production, an event that occurs during CH (Peers, 2002). As such, models have been proposed in which mitochondrial or NADPH oxidase derived ROS are thought to play a role in O_2 sensing and gene regulation during CH. Numerous studies have provided evidence that pharmacological inhibition of complexes in mitochondria or NADPH oxidase that produce ROS decreased the expression of genes that are regulated by CH (Fandrey *et al.*, 1994; Gleadle *et al.*, 1995; Ehbelen *et al.*, 1998).

The goal of this study was to examine the role of mitochondria and NADPH oxidase on the response of L-type Ca^{2+} channel α_{1C} subunits to CH. Drugs that inhibit these complexes, such as rotenone (a mitochondrial complex I inhibitor) and DPI (an NADPH oxidase inhibitor) were applied to cells in conjunction with 24 h incubation of cells under normoxic or chronically hypoxic conditions. Results show that mitochondrial complex I plays a significant role in mediating the CH response of recombinant L-type Ca^{2+} channels.

METHODS AND MATERIALS

Much of the same methodology was used as indicated in Chapter 1 under Methods and Materials. There were only a few adjustments that were made, which included using a different incubator and different drug applications.

Chronic hypoxia

To incubate cells under chronically hypoxic conditions, they were placed in a separate incubator equilibrated with an atmosphere of 6 % O_2, 5 % CO_2 and 89 % N_2 for 24 h prior to electrophysiological recording. Incubator temperature was held constant at 37° C.

Drug Solutions

Rotenone – Rotenone (Sigma) was stored in the freezer in 1 mM aliquots in DMSO. 2 μL of the aliquot was added to 2 mL of media for a final concentration of 1 μM rotenone. 2 ml of media was added to a dish of plated cells 24 h prior to recording and either incubated in normoxic conditions (Po_2 ~ 150 mmHg) or under chronic hypoxic conditions (Po_2 ~ 40 mmHg) depending on the experiment.

Diphenylene iodonium (DPI) – 1 μL of a 10 mM stock solution of DPI in DMSO was added to 2 ml of media for a final concentration of 5 μM. 2 ml of media was added to a dish of plated cells 24 h prior to recording and either incubated in normoxic conditions (Po_2 ~ 150 mmHg) or under chronically hypoxic conditions (Po_2 ~ 40 mmHg) depending on the experiment.

RESULTS

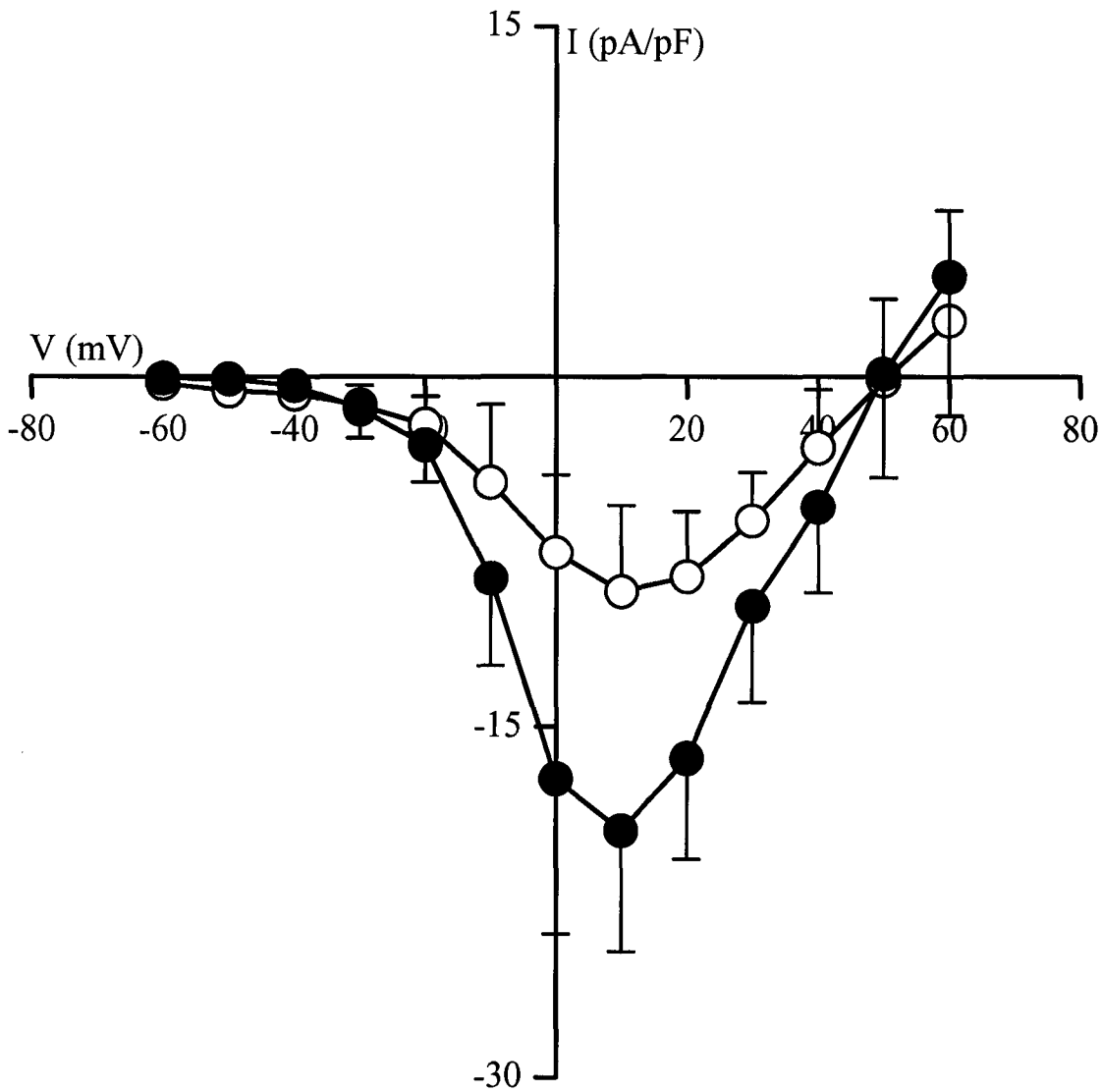
Chronic hypoxia enhanced Ca^{2+} currents through recombinant L-type Ca^{2+} channels

The purpose of this experiment was to determine the effect of prolonged exposure to hypoxia (chronic hypoxia; CH) on recombinant L-type Ca^{2+} channel α_{1C} subunits. Previous findings have demonstrated that Ca^{2+} currents become enhanced after exposure to chronic hypoxia due to selective up-regulation of L-type Ca^{2+} channels (Green and Peers, 2001). Cells were placed in an incubator with an atmosphere of 6 % O_2 for 24 h prior to making electrophysiological recordings. Figure 17 demonstrates that CH enhanced Ca^{2+} currents at all test potentials examined. For example, currents were enhanced from -9.15 ± 3.74 pA/pF in control cells (n = 11) to -19.41 ±5.18 pA/pF in cells incubated under CH conditions (n = 12, $P < 0.05$, unpaired Student's *t*-test). At +30 mV, current was enhanced from -6.1 ± 2.07 pA/pF in control cells to -9.79 ± 4.16 pA/pF in cells incubated in CH (n = 12, $P > 0.05$, unpaired Student's *t*-test). Thus, CH significantly enhanced Ca^{2+} current through L-type Ca^{2+} channel α_{1C} subunits.

NADH dehydrogenase regulated the chronic hypoxic response

It has been shown previously that rotenone, a mitochondrial complex I inhibitor, inhibited glomus cell O_2-regulated K^+ channel responses to acute hypoxia (Wyatt and

Figure 17: Chronic hypoxia enhanced L-type Ca^{2+} currents. Mean (± S.E.M) current density-voltage relationships obtained from cells incubated under normoxic conditions (Po_2 ~ 150 mmHg) (○, n = 11) and in chronically hypoxic conditions (Po_2 ~ 40 mmHg) (●, n = 12). Currents were evoked by step depolarizing cells for 100 ms to indicated test potential at a frequency of 1 Hz. Current (I, pA/pF) density was obtained by dividing the evoked current by the cell capacitance.


15
I (pA/pF)
V (mV)
-80
-60
-40
20
40
60
80
-15
-30

Buckler, 2004) and also regulated the release of neurotransmitters from these cells (Ortega-Saenz *et al.*, 2003). This experiment tested the involvement of mitochondrial complex I in the response of recombinant L-type Ca^{2+} channels to chronic hypoxia. As shown in Figure 18, application of 1 μM rotenone to cells for 24 h under normoxic conditions had no effect on Ca^{2+} channel currents. At test potentials at which currents were maximal, Ca^{2+} currents in normoxic control cells were -9.15 ± 3.74 pA/pF (n = 11) and -10.04 ± 4.63 pA/pF in cells treated with rotenone (n = 8, $P > 0.05$, unpaired Student's *t*-test). In contrast, when cells were treated with 1 μM rotenone under chronically hypoxic conditions, Ca^{2+} current enhancement due to chronic hypoxia was inhibited at all test potentials examined (Figure 19). For example, at 0 mV the mean currents were -22.97 ± 3.74 pA/pF in control (CH) cells (n = 12) and -9.37 ± 3.11 pA/pF in cells incubated under CH conditions in the presence of rotenone (n = 8, $P < 0.05$ unpaired Student's *t*-test). At +30 mV, mean control (CH) currents were -11.97 ± 3.3 pA/pF and were -5.56 ± 3.5 pA/pF for chronically hypoxic cells incubated with rotenone ($P > 0.05$, unpaired Student's *t*-test). Mean Ca^{2+} current magnitudes were significantly different between cells incubated in chronic hypoxia in the absence and presence of rotenone (Figure 20). These data indicated that rotenone inhibited Ca^{2+} current enhancement due to CH, and inferred that regulation of NADH dehydrogenase mediated the response to CH in cells expressing recombinant L-type Ca^{2+} channel α_{1C} subunits.

Figure 18: Effect of 1 μM rotenone on Ca^{2+} current through recombinant L-type Ca^{2+} channels. Mean (± S.E.M) current density-voltage relationships obtained from cells incubated under normoxic conditions (Po_2 ~ 150 mmHg) for 24 h prior to recording in the absence of rotenone (○, n = 11) and in the presence of rotenone (●, n = 8). Currents were evoked by step depolarizing cells for 100 ms to indicated test potential at a frequency of 1 Hz. Current density (I, pA/pF) was obtained by dividing the evoked current by the cell capacitance.

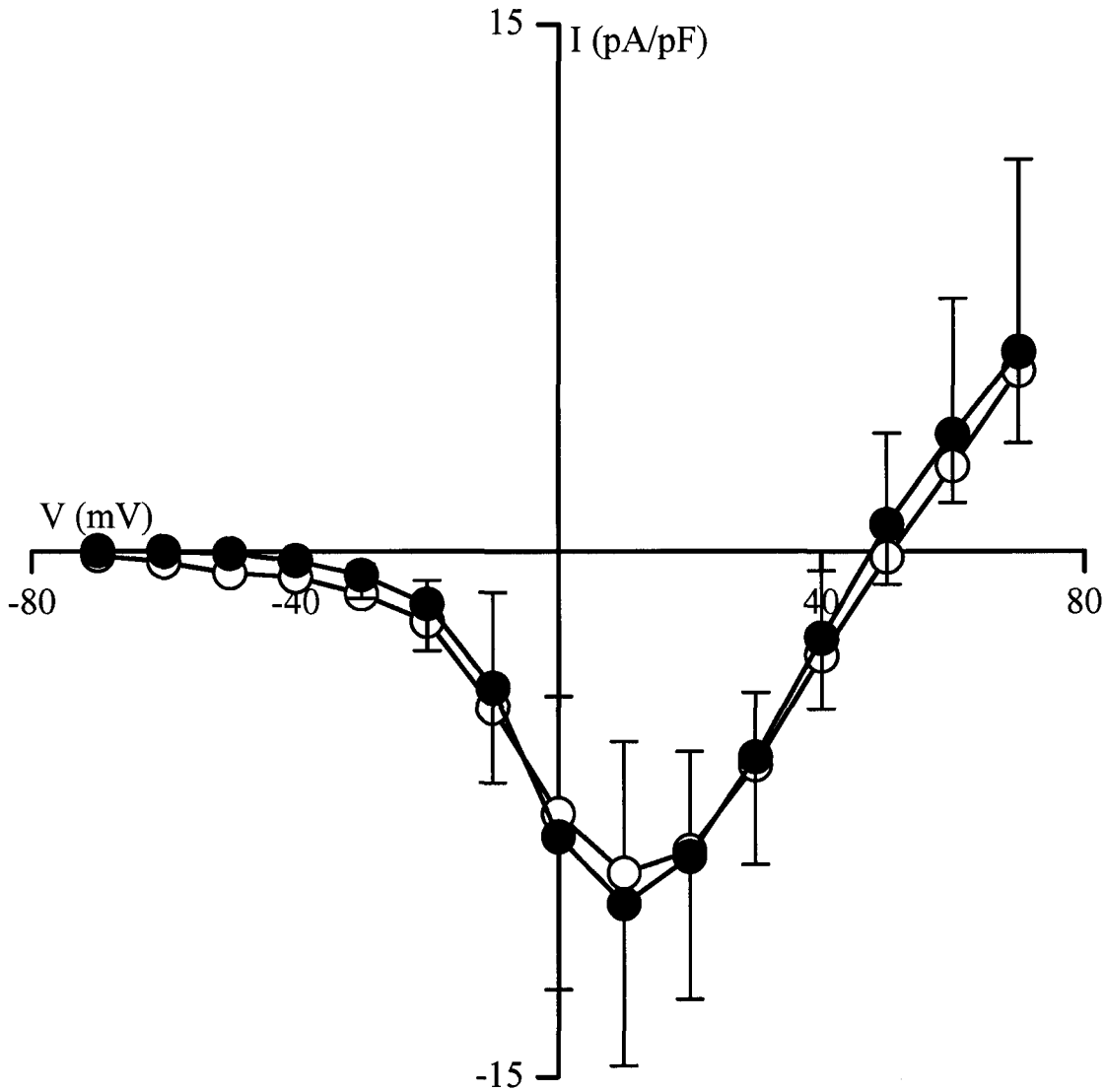

15
I (pA/pF)
V (mV)
-80
-40
40
80
-15

Figure 19: 1 μM rotenone inhibited chronic hypoxia-induced enhancement of Ca^{2+} current through recombinant L-type Ca^{2+} channels. Mean (± S.E.M) current density-voltage relationships obtained from cells incubated in chronically hypoxic conditions (Po_2~ 40 mmHg) in the absence (O, n = 12) or presence of rotenone (●, n = 8). Currents were evoked by step depolarizing cells for 100 ms to indicated test potentials at a frequency of 1 Hz. Current density (I, pA/pF) was obtained by dividing the evoked current by the cell capacitance.

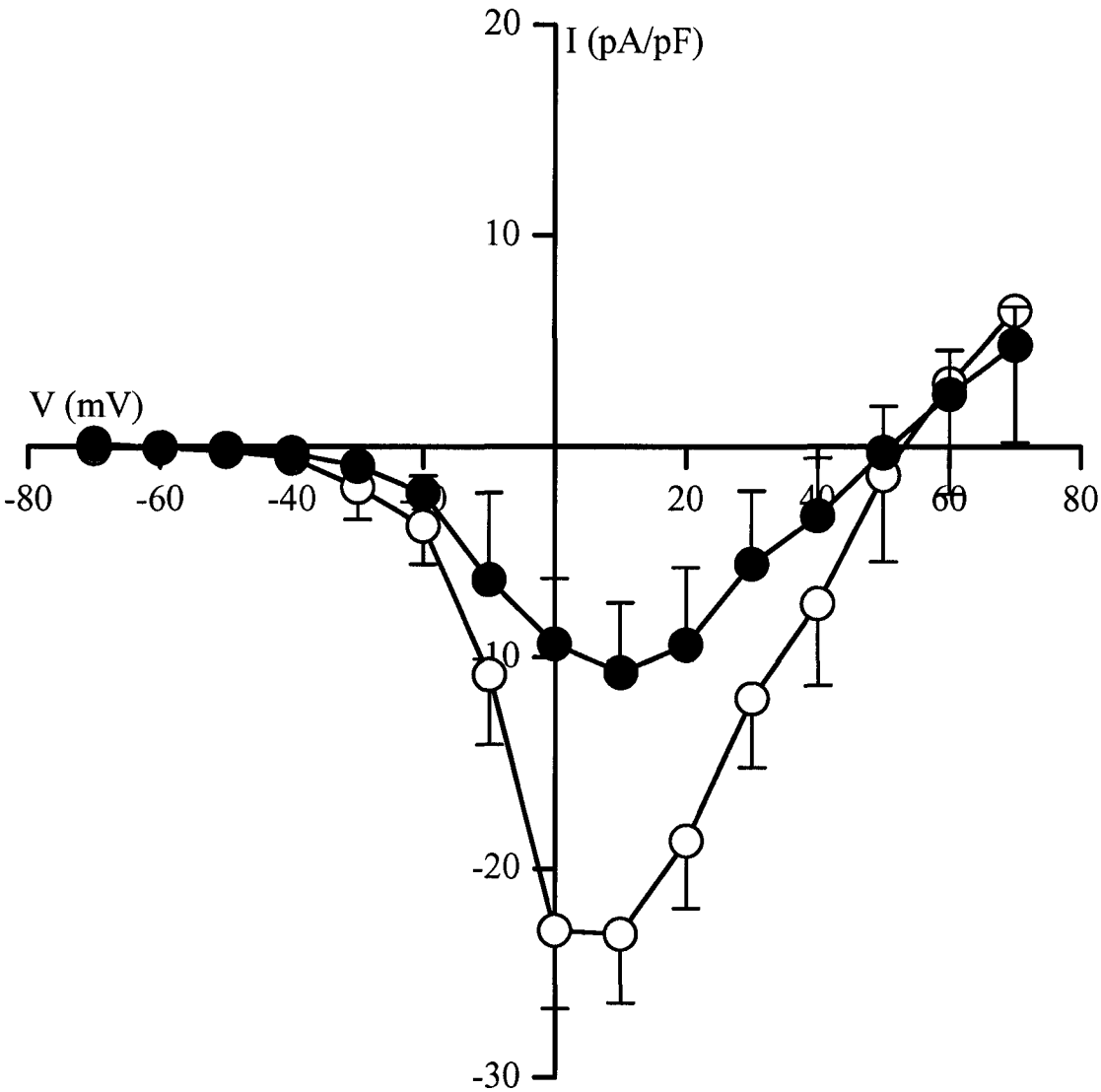
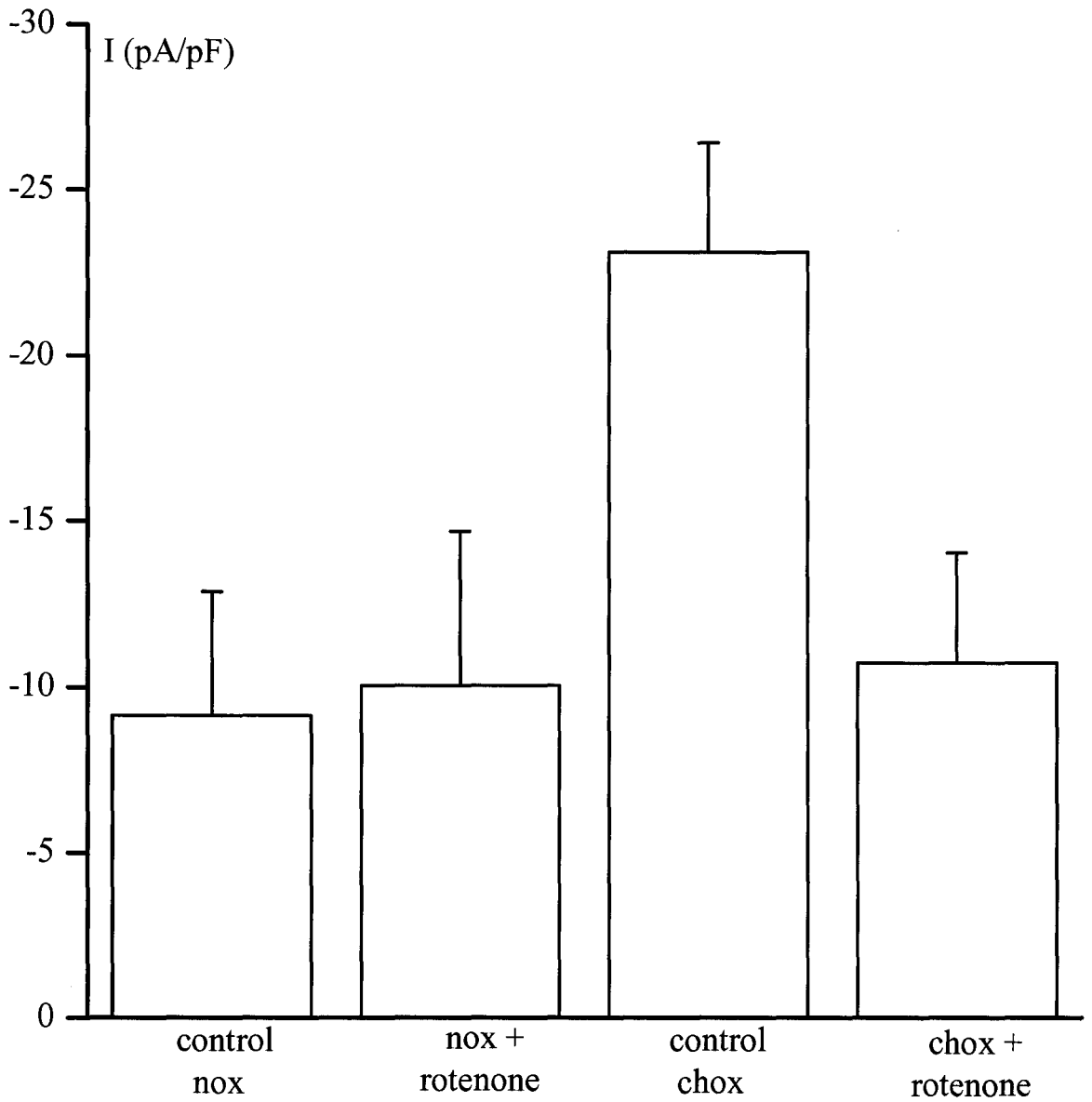

20
I (pA/pF)
10
V (mV)
-80
-60
-40
20
40
60
80
-10
-20
-30

Figure 20: Magnitude of Ca^{2+} current under normoxic and chronically hypoxic in the presence or absence of 1 μM rotenone. Data are plotted as means (± S.E.M) from recordings for normoxic controls (control nox, n = 11), chronically hypoxic controls (control chox, n = 12), normoxic cells with rotenone (nox + rotenone, n = 8) and chronically hypoxic cells with rotenone (chox + rotenone, n = 8). Mean Ca^{2+} currents at +10 mV were obtained from recordings such as those in Figure 15 and 16, and are shown in pA/pF following normalization for cell size by dividing current by the cell capacitance.


-30
I (pA/pF)
-25
-20
-15
-10
-5
0
control
nox
nox +
rotenone
control
chox
chox +
rotenone

NADPH oxidase did not mediate the response to chronic hypoxia of α_{1C} subunits

Above, I demonstrated that NADPH oxidase did not mediate the response of recombinant L-type Ca^{2+} channels to acute hypoxia. However, it is possible that this oxidase plays a role in the response to CH. Figure 21 shows that incubation of HEK 293 cells for 24 h with the NADPH oxidase inhibitor DPI under normoxic conditions was without effect on Ca^{2+} channel currents. For example, at a test potential of +10 mV, mean current densities were -9.15 ± 3.74 pA/pF in control cells ($n = 11$) and -10.81 ± 5.58 pA/pF in cells incubated with 5 μM DPI ($n = 7$, $P > 0.05$ unpaired Student's *t*-test). Similarly, cells incubated with 5 μM DPI under chronically hypoxic conditions with showed similar Ca^{2+} current amplitudes compared to control cells incubated in the absence of DPI under chronically hypoxic conditions (Figure 22). For example, at a test potential of +10 mV, currents were -19.42 ± 5.18 pA/pF in control (CH) cells ($n = 12$) and -21.44 ± 8.73 pA/pF in cells incubated with DPI under chronically hypoxic conditions ($n = 7$, $P > 0.05$ Student's unpaired *t*-test). Enhancement of Ca^{2+} currents due to CH was not abrogated by treatment with DPI as indicated in Figure 23, which shows current density-voltage relationships in cells subjected to normoxia, CH, and CH in the presence of DPI. These results indicated that DPI was without effect on basal Ca^{2+} current amplitudes and responses to CH and further suggests that regulation of NADPH oxidase does not mediate the response of L-type Ca^{2+} channel α_{1C} subunits to CH.

Figure 21: Long-term incubation with 5 μM diphenylene iodonium (DPI) had no significant effect on Ca^{2+} currents through recombinant L-type Ca^{2+} channel α_{1C} subunits. Mean (± S.E.M) current density-voltage relationships obtained for cells incubated under normoxic conditions without (○, n = 11) or with 5 μM DPI (●, n = 7) for 24 h prior to recording. Currents were evoked by step depolarizing cells for 100 ms to indicated test potentials at a frequency of 1 Hz. Current density (I, pA/pF) was obtained by dividing the evoked current by the cell capacitance. Data are presented as means (± S.E.M).

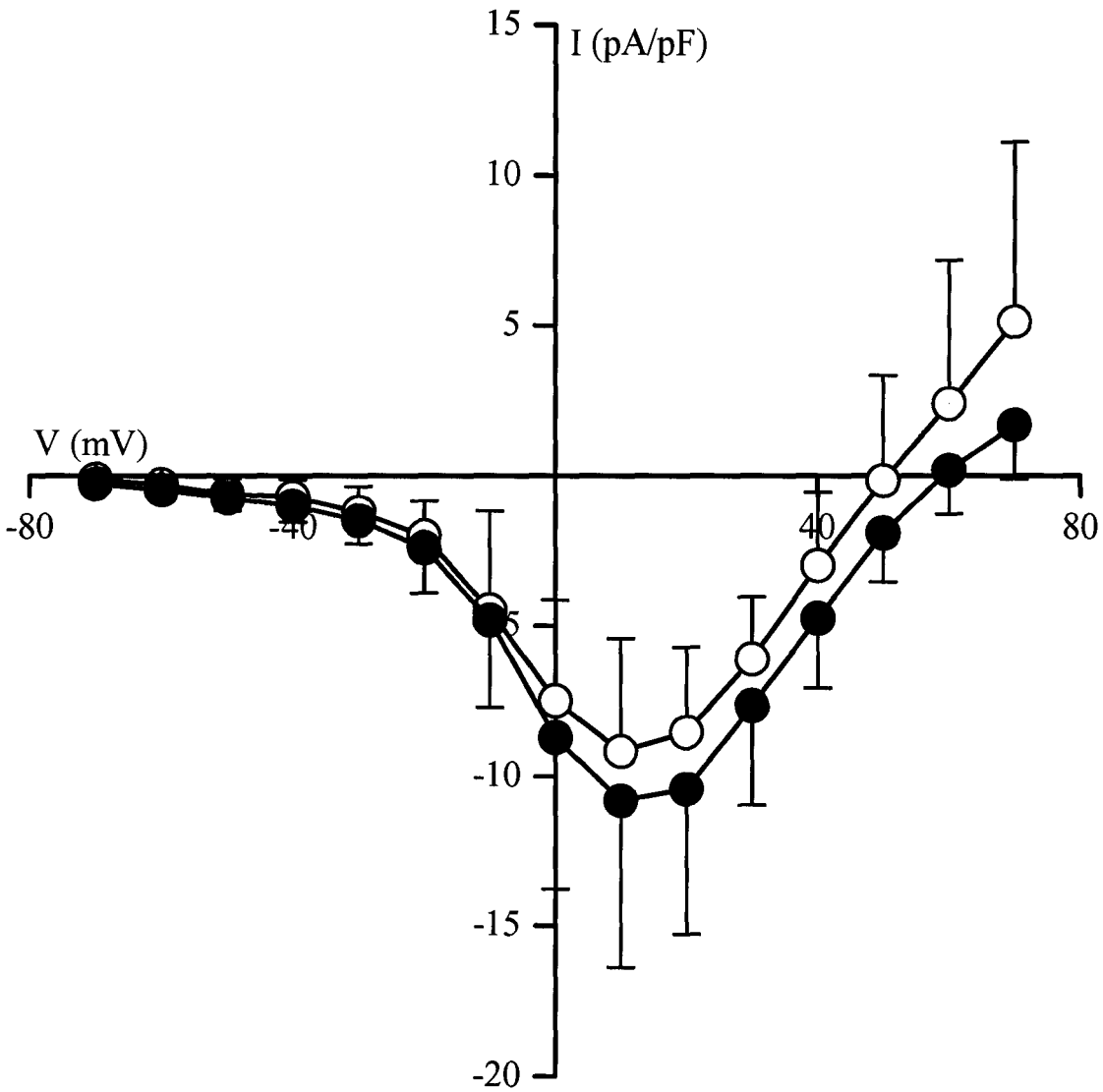
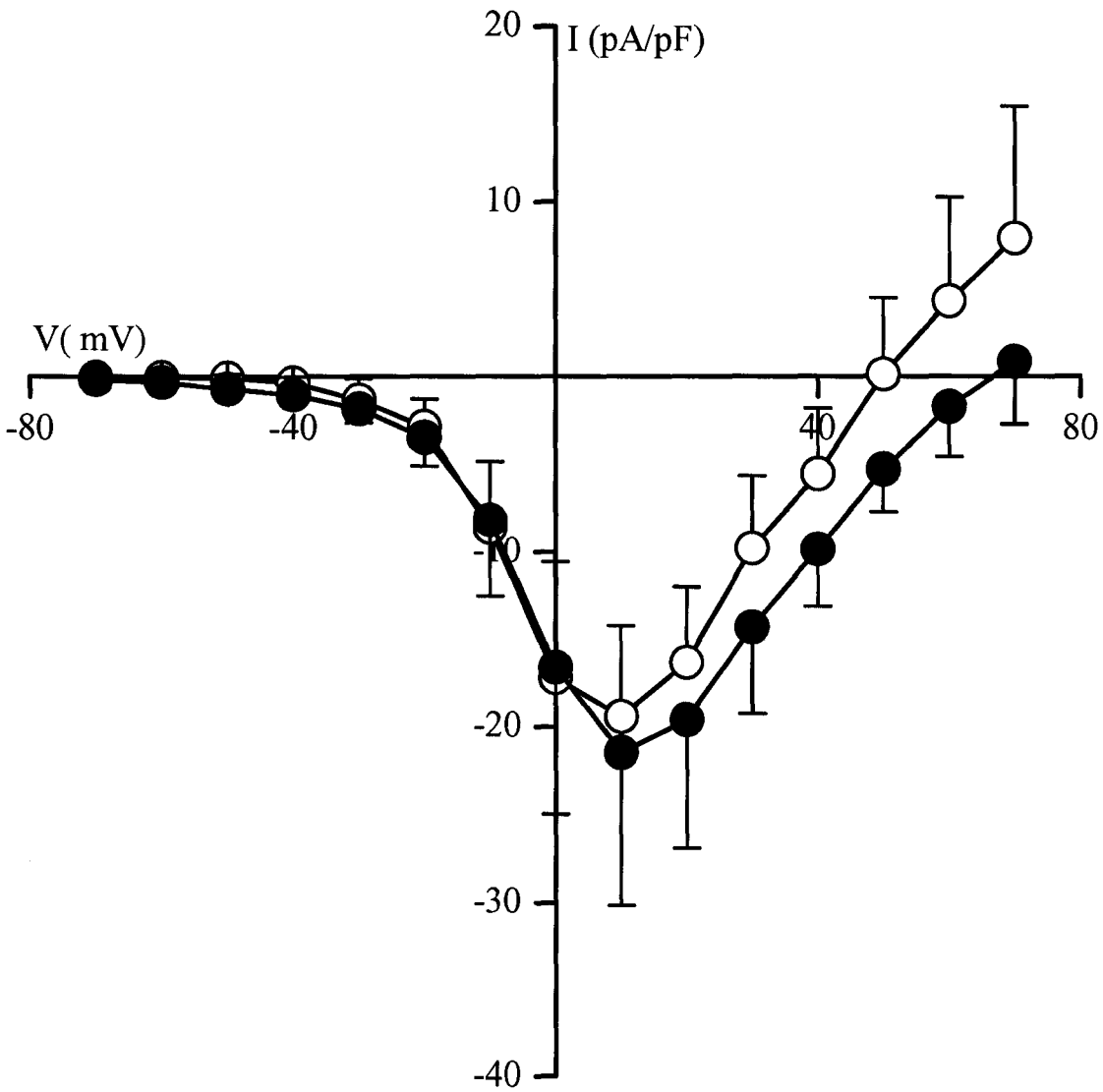

15
I (pA/pF)
10
5
V (mV)
-80
-40
40
80
-10
-15
-20

Figure 22: DPI had no effect on Ca^{2+} current enhancement due to chronic hypoxia (Po_2 ~ 40 mmHg). Mean (± S.E.M) current density-voltage relationships obtained in cells incubated in the absence (○, n = 12) or presence of 5 μM DPI (●, n = 7) under chronically hypoxic conditions for 24 h prior to recording. Currents were evoked by step depolarizing cells for 100 ms to indicated test potentials at a frequency of 1 Hz. Current density (I, pA/pF) was obtained by dividing the evoked current by the cell capacitance.


20
I (pA/pF)
10
V(mV)
-80
-40
40
80
-20
-30
-40

DISCUSSION

In recent years, there has been much investigation into how prolonged periods of hypoxia alter cellular function. Although most people do not normally experience chronically hypoxic conditions, diseases do exist that cause individuals to receive inadequate amounts of O_2 for extended periods of time. These diseases include chronic obstructive pulmonary disorder, asthma, pulmonary fibrosis and sleep apnea among others. Prolonged periods of hypoxia have been linked to Alzheimer's disease and other types of dementias (Moroney *et al.*, 1996; Bazan *et al.*, 2002; Peers *et al.*, 2004). Inadequate supply of O_2 to the brain can result from strokes, aneurysms and other diseases where tiny blood clots obstruct the vasculature and diminish the supply of blood to that area of the brain (Ramp, 1999). Unlike acute hypoxia, which affects existing ion channels, chronic hypoxia may alter the expression of various ion channels and/or auxiliary subunits (Hartness *et al.*, 2003). Under chronic hypoxia, rat pulmonary smooth muscle cells were found to be more depolarized compared to cells under normoxic conditions (Smirnov *et al.*, 1994). This depolarization was attributed to selective down-regulation of delayed rectifier K^+ channels. Similarly, voltage-gated K^+ channel α subunits from pulmonary artery smooth muscle cells of rats displayed inhibited gene expression when subjected to chronically hypoxic conditions (Wang *et al.*, 1997). Voltage-gated K^+ channel are important for setting the resting membrane potential and alteration of their expression is a major contributor to pulmonary hypertension and vascular remodeling (Wang *et al.*, 1997). Down-regulation of these voltage-gated K^+

channels would mean that these cells would have a more depolarized potential and thus be more electrically excitable. Although much is known about K^+ channel regulation during chronic hypoxia, relatively few studies have examined the regulation of the chronic hypoxic response of Ca^{2+} channels. Peers' group (1996) demonstrated that Ca^{2+} currents were enhanced under chronically hypoxic conditions compared to control (normoxic) conditions in rat carotid body type I cells. Del Toro and colleagues demonstrated that under chronically hypoxic conditions, T-type Ca^{2+} channel currents were enhanced in parallel with accumulation of mRNA encoding for the α_{1H} pore-forming subunit of this channel (Del Toro *et al.*, 2003). Previous finding have shown that Ca^{2+} current recordings in model neuroendocrine pheochromocytoma (PC12) cells subjected to CH were much greater in amplitude than control cells in normoxic conditions (Green and Peers, 2001). These data are in agreement with findings of this thesis. Ca^{2+} currents through L-type Ca^{2+} channel α_{1C} subunits was found to greatly increase in amplitude in HEK 293 cells subjected to 6% O_2 for approximately 24 h. In Green and Peers study, enhancement of current was due to selective upregulation of L-type Ca^{2+} channels although this parameter was not examined in this thesis. It is possible that enhanced current was due to an increase in cell size as observed in other previous studies (Peers *et al.*, 1996). However, cell capacitances between chronically hypoxic and normoxic cells in this thesis were not significantly different (data not shown). Thus, cells exposed to CH were shown to increase Ca^{2+} current amplitudes and alteration of Ca^{2+} homeostasis. The regulation of expression of the gene encoding for

this channel was not investigated in my thesis and as such, it cannot be concluded that Ca^{2+} currents increase were in fact due to an upregulation of channel-encoding genes.

It has been proposed that after chronic hypoxic episodes, during reoxygenation, cells increase production of ROS, which leads to oxidative stress and could contribute to diseases that then manifest as dementias (Prabhakar, 2001). Impaired mitochondrial function and ROS production can occur after prolonged periods of hypoxia and is also a hallmark abnormality in patients with Alzheimer's disease (de la Monte *et al.,* 2000). It has also been shown that components of the mitochondrial ETC such as complex IV and to a lesser extent, complexes I and II-III are decreased in brain tissue samples from Alzheimer's patients (Mutisya *et al.,* 1994, Buchner *et al.,* 2002). The mechanism by which CH causes impaired mitochondrial function is not fully understood but is thought to involve changes in redox state and altered ROS production which is thought to lead to altered gene expression. ROS is thought to play an important role in the potentiating effects of CH of L-type Ca^{2+} channels in PC12 cells (Green *et al.*, 2002). As CH is thought to affect mitochondria and thus ROS production by mitochondria, the role of complex I in the mitochondrial electron transport chain was examined. Complex I is a major contributor of ROS due to inefficiency in the ETC at this site. In HEK 293 cells, the mitochondrial complex I inhibitor rotenone, was found to abolish the enhancement of Ca^{2+} current due to CH while not affecting Ca^{2+} current amplitudes in control (normoxic) cells. Rotenone is thought to shift the cellular redox potential to a more reduced state as blockage occurs at the complex I (NADH dehydrogenase) such that NADH cannot be

oxidized to NAD^+. According to these data, a reduced cellular state could somehow affect channel kinetics so as to inhibit the Ca^{2+} current enhancement seen after exposure to CH. Rotenone has previously been found to decrease ROS production (measured by chemiluminescence) and increase pulmonary arterial pressure (Archer, 1993). It has also been shown that a bolus injection of rotenone decreased ROS production and increased vascular tone in isolated perfused lungs (Reeve *et al.,* 2001b). According to Reeve's model, complex I of the mitochondrial ETC acts as an O_2 sensor by maintaining a basal level of O_2^- and H_2O_2 during normoxia and that alterations in this basal level by rotenone would increase NADH leading to a more reduced cellular state and would inhibit K^+ channel current. This inhibition of K^+ current is similar to that seen during CH. It is important to note that rotenone was not found to mimic the effect of CH on Ca^{2+} currents but abolished the chronically hypoxic response of recombinant Ca^{2+} channels. This could mean that rotenone is altering redox status of the cell such that L-type Ca^{2+} channel currents are no longer enhanced by CH and are not affected during normoxia. This could be explained if there were alternate sources of ROS produced from sites other than complex I so as to not alter channel activity during normoxia but only during CH when the amount of available O_2 is decreased for these other ROS producing enzymes. It has been shown in models involving lungs of rats with chronic hypoxic pulmonary hypertension that pulmonary artery smooth muscle cells are in a more reduced state than normoxic control rats (Reeve *et al.*, 2001b). The reduced state inhibited BK_{Ca} channel and is thought to involve the ratio of NADH to NAD^+. Adding dehydropiandrosterone (DHEA), which has been suggested to induce decreases in the NADH: NAD^+ ratio

leading to oxidization of the cells (Gupte *et al.*, 2002), reversed the chronically hypoxic response of these cells by activating K^+ channels. Thus, the reduced state of the cell caused by CH is reversed by DHEA by activating K^+ channels and returning the cells to a more oxidized state. Thus, it appears that mitochondrial complex I mediates the alterations in redox state of the cell during CH. Altering the levels of reduced compounds regulated by this complex or inhibiting the complex itself has been shown both in K^+ channels and recombinant L-type Ca^{2+} channel α_{1C} subunits, to negate the affects of CH on ion channels.

NADPH oxidase is another major source of ROS that is thought to play a possible role in O_2 sensing. Although much examination has been completed in recent years that have investigated the role of NADPH oxidase in acute O_2 sensing, there has not been much investigation into the role of NADPH oxidase in regulating the response of various ion channels to CH. Previous studies have used DPI, a NADPH oxidase inhibitor, to study the effects of NADPH oxidase on gene expression during CH and have found that alteration of NADPH oxidase significantly inhibited the chronic hypoxic regulation of O_2-regulated genes (Gleadle *et al.*, 1995). I found that DPI was without effect on enhancement of Ca^{2+} currents due to CH. No significant difference was seen in current amplitudes of HEK 293 cells in the presence or absence of DPI under normoxic conditions or in the presence or absence of DPI under chronically hypoxic conditions. Thus, inhibition of NADPH oxidase did not regulate the chronic hypoxic response of L-type Ca^{2+} channel α_{1C} subunits. Other studies have shown that alteration of NADPH

oxidase did not effect hypoxic induction of gene expression in cells lacking a functional NADPH oxidase (Wenger *et al.*, 1996). Genes examined in this study, vascular endothelial growth factor (VEGF) and aldolase, which are known to be up-regulated during CH (Tipoe and Fung, 2003). These data concur with the aforementioned study in which NADPH oxidase was without effect on the chronic hypoxic response of L-type Ca^{2+} channels, either by not directly affecting channel activity via abrogation of current enhancement.

In summary, CH was found to enhance Ca^{2+} currents through L-type Ca^{2+} channel α_{1C} subunits and this enhancement was most likely due to alterations in the redox state of recombinant channels. Rotenone was found to abrogate enhancement of Ca^{2+} current due to CH while DPI did not regulate the chronic hypoxic response of L-type Ca^{2+} channel α_{1C} subunits. These data support the theory that mitochondria and/or complex I-derived ROS act as second messengers in regulating Ca^{2+} current enhancement during CH. The exact mechanism by which ROS transduces signals that modify O_2 sensitive Ca^{2+} current enhancement are not understood at this time. Further investigation into alteration of redox states will hopefully provide further insight into the mechanisms that underlie channel modification and vascular remodeling during CH.

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