Instituto Juan March de Estudios e Investigaciones

61 CENTRO DE REUNIONES INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Oxygen Regulation of Ion Channels and Gene Expression

Organized by

E. K. Weir and J. López-Barneo

H. Acker
S. L. Archer
P. Boistard
J. Caro
M. F. Czyzyk-Krzeska
L. L. Dugan
A. Franco-Obregón
C. González
G. G. Haddad
J. López-Barneo
C. A. Nurse
C. Peers
O. Pongs
P. J. Ratcliffe
H. Sies
N. B. Standen
B. L. Taylor
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INTRODUCTION

E. Kenneth Weir and José López-Barneo

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OXYGEN REGULATION OF ION CHANNELS AND GENE EXPRESSION

E. Kenneth Weir and José López-Barneo
Department of Medicine, VA Medical Center & University of Minnesota, USA and Departamento de Fisiología Médica y Biofísica, Universidad de Sevilla, Spain

This workshop concentrated on a relatively new but rapidly growing research field. The influence of O_2 sensing on cellular functions is an emergent topic that in the coming years will certainly have a broad impact in biomedicine. The initial progress in the field has been achieved independently by researchers with different background and technical expertise, without much communication among them. Thus, we thought that the gathering of a selection of scientists, representative of the various disciplines with interest in the subject, would render an enormous conceptual enrichment and could lead to a breakthrough in the maturation and development of the field. This was among the first international workshops on the subject aimed at the interchange of ideas and concepts among scientists from areas as diverse as molecular biology, biochemistry, physiology, pharmacology and clinical research.

Oxygen, one of the most abundant elements in the biosphere, is crucial for the maintenance of most life forms on earth. It has a major biological role as acceptor of the electrons in the mitochondrial respiratory chain and in doing so enables the synthesis of ATP by phosphorylative oxidation. Despite its paramount importance, little is known about how organisms are capable of sensing O_2 availability and adjusting the gas uptake to their changing needs, in different habitats or physiological situations. Because, in mammals, O_2 is taken up in the respiratory system and transported to the tissues by the blood, the most immediate adaptative response to the lack of environmental O_2 is an increase in the frequency of breathing. Acute hypoxia also produces dilatation in most arteries, which is an important mechanism participating in the local regulation of vascular tone. Besides these fast physiological responses, long-term hypoxia can induce in specific cells modifications in gene expression and enzymatic activity. Well-known examples of these chronic adaptations to the lack of O_2 are the induction of erythropoietin, the hormone that stimulates the production of red blood cells, and of vascular endothelial growth factor, which may mediate hypoxia-initiated angiogenesis.

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Research in recent years has begun to shed light on the basic cellular and molecular mechanisms underlying acute and chronic adaptations to low O_2 tension. The cardiorespiratory responses to hypoxia seem to be mediated by O_2-sensitive ion channels, expressed in glomus cells of the carotid body (the primary O_2-sensitive arterial chemoreceptors), arterial smooth muscle cells, neuroepithelial bodies of the lung, pheochromocytoma and brain cells. The molecular nature of the intrinsic O_2-sensors associated with ion channels, or those O_2-sensitive molecules capable of triggering the signal pathway(s) regulating transcription, although unknown, is currently being investigated in several laboratories. The molecular characterization of the O_2 sensitive molecules will surely lead to a better understanding of many pathophysiological processes (such as hypertension or the responses of brain and heart cells to ischemia) and will generate new strategies for the pharmacological treatment of human diseases.

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Session 1. CELLULAR AND MOLECULAR BASIS OF OXYGEN SENSING

Chairperson: Jaime Caro

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CELLULAR METABOLISM OF OXYGEN

Helmut Sies
Institut für Physiologische Chemie I, Heinrich-Heine-Universität Düsseldorf, Postfach 101007, D-40001-Düsseldorf, Germany
email: helmut.sies@uni-duesseldorf.de

General

The bulk of cellular oxygen uptake is utilized for energy production in the form of ATP by cytochrome c oxidase in the mitochondrial respiratory chain. The net 4-electron reduction of molecular oxygen to water occurs without release of intermediate radical species. This means that well over 95 % of total oxygen uptake occurs without the formation of reactive oxygen species (1). The mitochondrial respiratory chain may generate superoxide anion radicals at the level of ubisemiquinone, a process potentially related to long-term damage to mitochondria. Mitochondrial DNA is afflicted by oxidation to an extent about 10-fold higher than genomic DNA.

Oxidants

There a several enzymatic sites in the cell where intermediate steps of oxygen reduction occur (2). The one-electron step, i.e. formation of the superoxide anion radical, occurs enzymatically, and the process of redox cycling is driven by reductases utilizing NADPH. This process is of interest in toxicology, being the basis of the toxicity of quinones and nitroaromatics (3).

Peroxynitrite: Defenses. Nitric oxide and superoxide can be generated by inflammatory cells, and the two radicals react rapidly to form peroxynitrite. This cytotoxic reactive oxygen species leads to DNA damage by oxidizing guanine and by causing single-strand breaks. In proteins, peroxynitrite can lead to tyrosine nitration, potentially interfering with phosphorylation/dephosphorylation signaling pathways. So far, there is no known enzymatic defense against peroxynitrite, and low-molecular-mass compounds such as ascorbate, cysteine and methionine have been shown to react with peroxynitrite.

Our recent work showed that peroxynitrite appears to react preferentially with selenium compounds. Ebselen, an antiinflammatory selenoorganic compound, reacts with peroxynitrite, forming the corresponding selenoxide (4) at a second-order rate constant of $2 \times 10^6\ M^{-1}\ s^{-1}$ (5), which is about 100-fold higher than the rate constant observed with the above-mentioned compounds. The selenoxide can be reduced back to the parent compound at the expense of GSH or other thiols, so that a steady-state line of defense can be maintained. Selenium-containing compounds protect plasmid DNA from single-strand breaks better than the corresponding sulfur-containing compounds; e.g., selenomethionine protected DNA from peroxynitrite-induced damage more efficiently than methionine (6). Likewise, nitration reactions were suppressed efficiently by seleno-compounds (7). We postulate, based on preliminary evidence, that defense against peroxynitrite is a novel function of selenoproteins such as GSH peroxidase (8).

Antioxidants and Oxidative Stress

The pattern of oxidants is matched by an array of antioxidants. The nature of the diverse antioxidants encompasses enzymes and small molecules, including vitamins such as ascorbate or vitamine E and micronutrients such as carotenoids (9,10). A disbalance

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in the oxidant/antioxidant equilibrium in favor of the oxidants, leading to potential damage, is termed 'oxidative stress' (11,12). Antioxidants are involved in disease mechanisms, and there is interest in employing antioxidants in therapeutic strategies (13).

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Reactive oxygen intermediates as mediators for regulating ion channel activity and gene expression during cellular oxygen sensing.

H.Acker, W.Ehleben, T.Porwol: Max Planck Institut für Molekulare Physiologie, Rheinlanddamm 201, D-44139 Dortmund, FRG

Cellular oxygen sensing is a highly conserved process in evolution, most likely developed by bacteria and plants with the first appearance of oxygen in the atmosphere to combat the toxic effect of reactive oxygen intermediates (ROI). Signal pathways are now very well described in these systems involving ROI as second messengers to regulate ion channel open probability as well as gene expression. The same processes seem to take place in mammalian cells using several ROI to up - or downregulate different genes. The main pathway seems to be the modifcation of transcription factors as well as the increase or decrease of membrane potassium currents by influencing the inactivation mechanism of the corresponding ion channel. The aim of our study is the identification of the oxygen sensor protein monitoring changes in oxygen partial pressure (PO_2) and of the intracellular localisation of different ROI for inciting the oxygen sensing signal pathway. We use carotid body - as well as HepG2 - cells for this purpose. These two cell systems are well known to regulate potassium ion channel actvity and gene expression in dependence on PO_2. We could identify as a putative oxygen sensor protein by means of light absorption photometry, immunohistochemistry and western blot analysis a low output cytochrome b with respect to ROI generation with components similar to the one as described for the high output neutrophil NADPH oxidase. The intracellular level of ROI in HepG2 cells is controlled by the glutathion peroxidase with an enhanced activity under hypoxia and cobalt application leading to an upregulation of the erythropoietin (Epo) and vascular endothelial growth factor (VEGF) gene. Furthermore, a local Fenton reaction observed in perinuclear structures of HepG2 cells by means of 3D confocal images seems to be involved explaining the upregulation of gene expression with application of the iron chelator desferrioxamine. This might hint to the importance of hydroxyl radicals downregulating Epo and VEGF expression in HepG2 cells under normoxia whereas primary hepatocytes as shown in the literature seem to use the same mechanism to upregulate the glucagon stimulated phosphoenolpyruvate carboxykinase gene (PEPCK). As described in literature potassium channels Kv 1.4, 3.3, 3.4 most likely expressed also in carotid body cells increase the potassium current under H_2O_2 application whereas $^1O_2$ inhibits this current. We believe based on these facts in an unifying concept of oxygen sensing valid for procaryots and eucaryots for regulation of ion channel activity and gene expression with distinct action of various ROI generated by hemeproteins as a function of PO_2.

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A critical cysteine residue in the inactivation domains of voltage-activated potassium channels

Olaf Pongs

Institut für Neurale Signalverarbeitung

Zentrum für Molekulare Neurobiologie

University of Hamburg, Germany

Voltage-activated potassium (Kv) channels are key players in many aspects of electrical responses in the nervous system. For example, Kv channel activities are involved in the regulation of wave forms and frequencies of action potentials, of thresholds of excitation, of synaptic strength, and of the resting potential of membranes. Two main types of Kv channels may be discerned: slowly or non-inactivating Kv channels and rapidly inactivating (A-type) Kv channels. A-type Kv channels may operate in the subthreshold range of action potentials and thereby fulfill important regulatory functions for encoding pre- and postsynaptic neural signalling. Several A-type Kv channel mediated currents have been described which differ in the voltage-sensitivity of activation and inactivation and/or in the time constants of activation, inactivation and recovery from inactivation. Thus, diverse A-type Kv channels are expressed in the nervous system suggesting that A-type Kv channels may have specialized and distinct functions related to nervous excitability.

The characterization of cloned A-type Kv channels has indicated that A-type Kv channels are most likely heteromultimers which are assembled from α and ß subunits. The primary sequences of Kv a subunit isoforms, which have been derived from many different cDNAs from bacteria and plants to man, are quite similar, always exhibiting a characteristic type of secondary structure. Accordingly, all Kv α subunit isoforms may have comparable structures consisting of hydrophobic, membrane-spanning core domains flanked by hydrophilic amino and carboxy termini of varying lengths. It appears that the core domain is inserted into the membrane such that the amino and carboxy termini face the cytoplasm. The core domain of Kv α subunits invariably contains six hydrophibic segments (S1 to S6) which transverse the membrane and one hydrophobic segment H5 interspersed between S5 and S6. Segment S5 is tucked into the membrane and enters and exits the plasma membrane from the extracellular side. The core domain of Kv α subunits contains the structures that are necessary to form the pore and the voltage-sensor which determines the voltage-sensisitivity of Kv channel opening. The cytoplasmic amino terminus contains the domains which are required for assembly and tetramerization (T-domains). Some carboxy-termini may contain a carboxyterminal signature sequence for interaction with the members of the postsynaptic density (PSD) protein family.

The ß subunits aooear to be peripheral proteins tightly associated with the cytoplasmic side of the a subunits. So far, only ß subunits have been cloned and characterized that interact with members of the Kv1α subunit subfamily. But indirect evidence suggests that the α subunit members of the other Kv1 subfamilies also associate with ß subunits. We showed the Kv1ß interaction site to be localized to a cytoplasmic amino terminal region within the tetramerization domain $T1_B$. Possibly, four Kv1ß subunits bind to Kv1α tetramers to form α4ß4 heteromultimers. Apparently, Kvß subunits are auxiliary subunits which may aid assembly of Kv α subunits and/or transport of the newly formed Kv channels to the plasma membrane. Another important function of Kvß subunits can be that they confer rapid inactivation onto Kv α channels which otherwise cannot rapidly inactivate, but behave like delayed rectifiers. This property is due to the presence of an amino terminal inactivating domain in some ß subunits like Kvß1 and Kvß3. The Kvß inactivating domain appears to be structurally related to the amino terminal inactivating domain which was previously identified in

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some Kv α subunits like Kv1.4 and Kv3.4. For Kv α subunits it has been shown that they contain a receptor for the inactivating domain near or at the inner mouth of the pore. Binding of the inactivating domain to this receptor site upon depolarization of the membrane rapidly closes the open Kv channel pore. It has been proposed that inactivating domain binding resembles a ball and chain type mechanism, in which the inactivating domain would be the ball and other parts of the amino terminus the chain.

Amino terminal Kv α and Kv β inactivating domains have several structural features in common. They do not require for their function a covalent bond to the rest of the molecule. If one adds to the bathing solution of inside-out patches inactivating domains in the form of peptides (approximately 25 amino acids long), noninactivating Kv channels are rapidly inactivated by binding the peptide to a receptor side located near or at the inner mouth of the Kv channel pore. Furthermore, the inactivating domains, which have been characterized so far, may be divided into two subdomains. One subdomain contains a relatively high density of positively charged amino acids. This positive charge density may be important for the binding reaction of the inactivating domain. The other subdomain contains a critical cystein residue which may be flanked by serine, alanine and/or other hydrophobic residues. This subdomain appears to determine the stability of the inactivating domain receptor interaction.

Interestingly, the inactivating domains which have been discovered in mammalian Kv α and Kvβ subunits all contain at a homologous sequence position a cysteine. This cysteine has to be maintained in a reduced condition for inactivating domain activity. When the cysteine residue has been oxidized, the inactivating domain can no longer close the Kv channel pore. Probably, this is due to a loss of binding activity. Importantly, the cysteine residues which are present in α subunit inactivating domains and those in Kvβ domains are both sensitive to oxidation. Thus, regardless whether the inactivating domain is bonded to a Kv α amino terminus or a Kvβ amino terminus, the activity of Kv inactivating domains is very sensitive to oxidation. Thereby, Kv channels rapidly inactivate when the cysteine is in a reduced state. By contrast, Kv channels do not rapidly inactivate when the cysteine is in an oxidized state. The oxidation process disabling the inactivating domain is rather fast but only involves those inactivating domains not bound to the inner mouth of the Kv channel. The sensitivity of the inactivating domains to oxidation is eliminated when cysteine in the inactivating domains is replaced by serine. Kv channels with this mutant inactivating domain still inactivate rapidly, but the inactivation time constants are now indistinguishable under oxidizing and reducing conditions. However, recovery from inactivation in the mutants is accelerated in comparison to wild type. The results suggested that serine replacement of the cysteine destabilizes the complex between the inactivating domain and the Kv channel pore. This observation may again underline the importance of a reduced cysteine for the stability of the complex between the inactivating domain and the Kv channel pore. In conclusion, rapid inactivation of mammalian A-type Kv channels is regulated by cysteine oxidation. This type of regulation may have a role in vivo to link the cellular redox status to the excitability of membrane areas containing A-type Kv channels.

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Cysteine proteinase gene expression in legume seed germination: a possible role for oxygen?.

Emilio Cervantes. Departamento de Biología Vegetal. Facultad de Biología. Universidad de Salamanca. E-37007. Salamanca. España.

By RT-PCR and subsequent cDNA library screening, a cysteine proteinase encoding cDNA was cloned from cotyledons of germinating garbanzo (*Cicer arietinum*, L.). The corresponding mRNA is first detected in northern blots several hours following seed imbibition, but not during seed development or in dry seeds, reaching a maximum between 48 and 96 hours after the begining of imbibition (1).

The embryonic axis is involved in regulating expression of this gene (termed cacG1 for *Cicer arietinum* germination) because in cotyledons imbibed after ablation of the embryonic axis, transcript levels are significantly reduced. When the cotyledons are imbibed after ablation of the embryonic axis and simultaneous removal of the testa, transcript levels increase to close to normal values during germination. This pattern of expression is similar to that previously reported for diamine oxidase activity in the cotyledons of pea (2) and suggests regulation by oxygen.

Also, mRNA levels for cacG1 are responsive to different concentrations of ethylene and oxygen under different experimental conditions, suggesting that ethylene and oxygen may have aspects in common in their signal-transduction chains.

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Session 2. OXYGEN SENSORS IN BACTERIA

Chairperson: Helmut Acker

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Nitrogen fixation and oxygen sensing

P. Boistard, E. Soupène and J. Batut
Laboratoire de Biologie Moléculaire des Relations Plantes-Microorganismes
CNRS- INRA, BP 27 31326 Castanet Tolosan Cedex Fr

Biological nitrogen fixation is performed by a variety of microoorganisms which belong to taxonomically distant genera. However the enzymatic complex nitrogenase which allows reduction of molecular nitrogen into ammonia is highly conserved, likely due to the stability of the triple bond which has to be disrupted and therefore the sophisticated chemical reaction to be performed.
Nitrogenase is made up of two protein components whose X ray structure has been determined in the recent years. Nitrogenase sensu stricto or component one is a hetero tetramer with two different polypeptide chains, alpha and beta. The coenzyme is an iron molybdenum complex. Transfer of electrons to nitrogenase is mediated by component two or nitrogenase reductase which is a homodimeric iron metalloenzyme. Nitrogenase reductase is particularly sensitive to oxygen.
Because nitrogen is involved in a major metabolism, nitrogenase can constitute a significant portion of cell proteins and its synthesis divert a major part of the cell resources. Therefore, its synthesis is repressed at oxygen concentrations deleterious for the enzyme.
In addition, because the energy requirement for nitrogen fixation is high, some nitrogen fixing bacteria couple nitrogen fixation with a highly efficient respiration process adapted to low oxygen tensions. The expression of the corresponding respiratory chain is also regulated by oxygen concentration.
Nitrogen fixing bacteria have adopted different regulatory cascades to regulate the expression of their nitrogen fixing apparatus according to the oxygen status. However, they often contain homologous regulatory proteins whose modulatory architecture allows a combinatorial organization of the signal transduction pathway. Oxygen sensing modules are present in different components of the cascades and illustrate different chemical possibilities for oxygen signal perception and transduction.
For example, most nitrogen fixing organisms possess a regulatory protein, NifA which controls the expression of nitrogen fixation genes in concert with the sigma 54 RNA polymerase holoenzyme. In most instances, NifA activity is intrinsically sensitive to oxygen whereas in *Azotobacter vinelandii*, oxygen is sensed by a flavoprotein NifL. Furthermore, in *Sinorhizobium meliloti*, the expression of *nifA* itself is controlled by a pair of regulatory proteins, one of which, FixL, is an oxygen binding hemoprotein which controls the activity of the transcriptional activator FixJ. In addition, FixL/J control the expression of the microaerobiosis-adapted respiratory chain through an Fnr homolog, FixK. In *Bradyrhizobium japonicum*, whereas FixK, similarly to *S. meliloti* controls the expression of the microaerobic respiratory chain, *nifA* expression is under an autoregulatory control.
Additional examples of this combinatorial organization associated with the modularity of the regulatory proteins, will be given.
In alfalfa nodules elicited by its symbiotic partner *S. meliloti*, the expression of the nitrogen fixing apparatus is coupled with nodule development. The oxygen-responsive regulatory cascade of nitrogen fixation gene expression is a major determinant of this coupling and will be shown to operate through several of its components.

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OXYGEN TRANSDUCERS REGULATE BEHAVIOR IN *ESCHERICHIA COLI*

Barry L. Taylor

Department of Microbiology and Molecular Genetics and Center for Molecular Biology and Gene Therapy, Loma Linda University, Loma Linda, CA 92350.

Behavioral responses to oxygen (aerotaxis) are ubiquitous in motile microorganisms. They have been studied for 100 years but the oxygen sensors have only recently been identified. Oxygen can act as both an attractant and a repellent. In a spatial gradient of oxygen, bacteria are attracted to a concentration of oxygen that is optimal for the metabolic lifestyle, and repelled by oxygen concentrations that are higher or lower than the preferred oxygen concentration. As a result, the bacteria form a focused band at the preferred oxygen concentration. Previous studies in this laboratory have demonstrated a requirement for a functional electron transport system for aerotaxis and indicated that the aerotaxis transducer detects and responds to a change in the electron transport system or in the proton motive force. Subsequently, we established that the CheA, CheW and CheY proteins are components of the signal transduction pathway for aerotaxis, in addition to chemotaxis in *Escherichia coli* (1). The chemotaxis pathway is a phosphorylation cascade in which the CheA sensor kinase and CheY cognate response regulator are members of the family of two component regulatory systems (2). The chemotaxis receptors Tsr, Tar, Trg and Tap span the cytoplasmic membrane and have a highly conserved signaling sequence in the cytoplasmic C-terminal domain which binds CheA and CheW and modulates autophosphorylation of CheA. We postulated that the aerotaxis transducer would have a domain that senses oxygen directly or indirectly, and a conserved signaling domain that modulates CheA autophosphorylation.

A BLASTP search identified an open reading frame at 69.1 minutes on the *E. coli* chromosome that had an N-terminal sequence with 47% similarity to the N-terminal domain of NifL and a C-Terminal domain with 96.7% identity to the signaling domain in Tsr. NifL is an oxygen-sensing protein of the NifL-NifA two-component system that regulates transcription of nitrogen fixation genes in *Azotobacter vinelandii* (3). The ORF in *E. coli* was renamed *aer*. Aerotaxis in an *aer-2::kan* mutant was reduced by one half and was restored by expression of *aer*

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from the pGH1 plasmid. Aerotaxis was abolished in a *tsr aer* double mutant. Aer and Tsr were shown to be two independent transducers for aerotaxis that mediate the tumbling response of *E. coli* to decreased oxygen and the smooth swimming response to an oxygen increase.

We have isolated Aer by nickel affinity chromatography and found that it is a flavoprotein with FAD as a prosthetic group. Tsr has cytoplasmic and periplasmic pH receptors. Our current hypothesis for oxygen sensing is that oxygen increases respiration in *E. coli*. Aer senses a change in redox state and Tsr senses a change in proton motive force. Aer and Tsr undergo conformational changes that decrease the autophosphorylation of CheA.

The Aer and Tsr proteins can also be considered as sensors of the internal energy state in *E coli*. They mediate a recently discovered redox taxis (4) and an energy taxis in addition to aerotaxis. Thus Aer and Tsr integrate different signals that relate to the energy state and guide *E. coli* to the micro environment that is optimal for energy production, growth and survival.

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Session 3. O_2 SENSING AND EUKARYOTIC GENE EXPRESSION (I)

Chairperson: Pierre Boistard

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Oxygen regulated gene expression in mammalian cells. P.J. Ratcliffe, G. Dachs, B.L. Ebert, J.D. Firth, J.M. Gleadle, P.H. Maxwell, J.F. O'Rourke, C.W. Pugh, I. Stratford, S.M. Wood. Institute of Molecular Medicine, John Radcliffe Hospital, Oxford. OX39DU.

Recent evidence has linked several groups of mammalian genes to what appears to be a common mechanism of gene regulation by oxygen working near the physiological range. Functional similarities in regulation have been observed, including responses to cobaltous ions and iron chelators as well as hypoxia. For a number of these genes, transfection and DNA binding studies have implicated the transcriptional complex hypoxia inducible factor-1 (HIF-1) in the inducible responses.

To define more clearly the role of HIF-1 or related complexes in the regulation of mammalian gene expression we have compared responses to hypoxia and iron chelation in wild type murine hepatoma cells (Hepa-1), and mutant derivatives (c4) which are defective for one component of the DNA binding heterodimer (HIF-1β), and unable to form a HIF-1 complex. In the mutant c4 cells defective responses to hypoxia were observed for a large number of genes, which includes examples where HIF-1 has previously been implicated by transfection studies (e.g. VEGF, Glut-1), as well as newly defined hypoxically regulated genes identified by differential display PCR. These responses clearly demonstrate the critical importance of HIF-1β in a widely operative system of gene regulation by oxygen. Equally, residual responses to hypoxia in HIF-1β deficient cells demonstrate the existence of HIF-1β independent regulatory mechanisms, which also respond to iron chelation, and which, for some genes, appear to be exaggerated in HIF-1β deficient cells.

To investigate this system of gene regulation in an *in vivo* context, we monitored tumour growth of Hepa-1 cells and the HIF-1β defective mutant derivatives, in xenotransplantation experiments. In keeping with the differences in inducible gene expression seen in tissue culture, substantial differences in tumour growth and vascularity were observed, indicating that this sytem is activated in hypoxic conditions arising within solid tumours, and demonstrating its importance in tumour biology.

To analyse the mechanism of HIF-1 activation by hypoxia, and the domain structure of the molecule, we have fused HIF-1 cDNAs to the DNA binding domains of heterologous transcription factors. HIF-1α but not HIF-1β chimeras were found to convey hypoxically inducible expression on either the glucocorticoid receptor or Gal4 DNA binding domains, a result which supports the existence of important post-translational control mechanisms for HIF-1α. Detailed analysis of these chimeras has defined at least 2 regions of the HIF-1α molecule which can convey oxygen dependent transcriptional activation and presumably interact with a signal transduction or sensing mechanism.

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EVIDENCE FOR HYDROGEN PEROXIDE AS A SIGNAL TRANSDUCTION MOLECULE IN OXYGEN DEPENDENT REGULATION OF GENE EXPRESSION.

Maria F. Czyzyk-Krzeska, Sandra L. Kroll, Waltke R. Paulding and Amy C. Bendixen
Department of Molecular and Cellular Physiology, University of Cincinnati, Cincinnati, OH 45267-0576, USA

Tyrosine hydroxylase (TH), the rate limiting enzyme in catecholamine synthesis, is induced by hypoxia in O_2-sensitive type I cells of carotid body and also in a clonal cell line PC12 that is used as a model system for carotid body cells. This induction involves increase in the amount of TH protein and TH mRNA. Our laboratory has shown that the O_2-dependent regulation of TH gene expression occurs at the level of TH gene transcription and TH mRNA stability. The O_2-sensitive mechanism that mediates regulation of TH gene expression by hypoxia is complex and potentially involves different signal transduction pathways that are affected by pO_2, such as for example membrane depolarization due to the presence of O_2-sensitive K^+ conductance, increase in the intracellular calcium concentration, or increase in the intracellular cGMP. Recently a novel and interesting evidence has been presented that physiological by-product of oxidative metabolism, **hydrogen peroxide (H_2O_2)**, participates in the O_2-sensing pathway in various O_2-sensitive cells. We have therefore tested whether hydrogen peroxide is involved in the response of PC12 cells to hypoxia. The measurements of H_2O_2 using 2'7'-dichlorodihydrofluorescein revealed that under normoxic conditions PC12 cells contain high amounts of H_2O_2. Exposure of cells to 5% O_2 led to 30% (n=4) and 50% (n=5) decrease in H_2O_2 measured after 3h and 20 h respectively. Levels of H_2O_2 were closely correlated with the expression of TH mRNA. Treatment of PC12 cells with a reducing antioxidant, N-(2-mercaptopropionyl)-glycine (NMPG, 10 mM) caused a dose dependent induction of TH mRNA that was identical in time course and magnitude to that measured when hypoxia was applied. NMPG reduced levels of H_2O_2 in PC12 cells by 20% (n=4) and 40% (n=4) after 3 and 20 h, respectively. Moreover, the effects of both hypoxia and NMPG could be completely counterbalanced by treatment of PC12 cells with aminotriazole (ATZ), catalase inhibitor, that resulted in recovery of H_2O_2 concentration to approximately 90% of the control value. Treatment of cells with deferoxamine (DF, 100 μM), iron chelator and thus hydroxyl radical scavenger, caused induction of TH mRNA similar to that measured during hypoxia, while when DF was applied simultaneously with hypoxia the increase in TH mRNA was abolished. Measurements of H_2O_2 during DF treatment revealed a 25% and 10% increases after 3h and a 20 h, respectively.

Next we examined whether H_2O_2 affects TH gene expression at the level of mRNA stability. We found that NMPG or DF had stabilizing effect on TH mRNA, very similar to that measured during hypoxia. The combined effects of hypoxia and DF were subtractive, and increase in H_2O_2 concentration with ATZ prevented hypoxia induced increase in TH mRNA stability. Our laboratory has previously shown that the O_2-dependent increase in TH mRNA stability is due to increased binding of a **hypoxia inducible protein** (HIP) to the cytidine-rich element within 3' untranslated region of TH mRNA **(HIPBS element)**. We found that the binding of HIP to the mRNA was redox dependent and involved reduced cysteine thiol groups. In addition, increase in reducing conditions in the *in vitro* binding reaction (using reducing agents such as 10-20 mM DTT) induced binding of the protein to the mRNA similarly to the induction caused by hypoxia. We thus examined effects of changes in H_2O_2 concentration on the binding of HIP to HIPBS. The binding was strongly induced by either NMPG or DF. These data support the hypothesis that hypoxia induced decreases in H_2O_2 and consequently hydroxyl radical concentrations lead to augmented number of the reduced thiol groups that in turn results in enhanced binding of the regulatory proteins to the nucleic acids and increase in gene expression.

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Session 4. O_2 SENSING AND EUKARYOTIC GENE EXPRESSION (II)

Chairperson: Colin Nurse

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Molecular mechanism in the erythropoietin response to hypoxia.

S.Salceda, Vickram Srivinas and Jaime Caro.
Cardeza Foundation for Hematologic Research.
Philadelphia, PA. U.S.A.

Erythropoietin (Epo) is the glycoprotein growth factor that controls red cell production and adapts the red cell mass to the oxygen requirements of the tissues. Erythropoetin is produced primaraiy in the kidney of adult mammals by a process involving transcriptional activation of the erythropoietin gene. This transcriptional activation is mediated by an hypoxia responsive enhancer element located at the 3' flanking region of the Epo gene. The recently identified hypoxia inducible factors α and β form a DNA-binding complex (HIF-1) that interact with the enhancer and induce transactivation. Messenger RNA levels of the two components of the complex are not significantly affected by hypoxia, suggesting a translational or post-translational control. However, the exact mechanisms involved in the formation of the DNA-binding complex and its transactivational activity are still poorly understood. Preliminary results from our laboratory suggest that protein phosphorylation is an important component in the transactivational activity of the HIF-1 complex.

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ACTIVATION OF HYPOXIA-INDUCIBLE FACTOR 1 DEPENDS ON STABILIZATION OF ITS α SUBUNIT

L. Eric Huang and H. Franklin Bunn

Hematology-Oncology Division, Brigham & Women's Hospital, Harvard Medical School, Boston, MA 02115, USA

Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric transcription factor which is activated in a wide range of cells exposed to hypoxia and is critical for the regulation of a number of physiologically important genes. HIF-1 is composed of a 120-kD α subunit and a 91-94 kD β subunit. Both subunits are members of the basic-helix-loop-helix-PAS family of transcription factors; HIF-1α is a novel protein whereas HIF-1β is the previously cloned and characterized aryl hydrocarbon receptor nuclear translocator. Here we present evidence that regulation of HIF-1 activity is primarily determined by the stability of HIF-1α protein. Both HIF-1α and HIF-1β mRNAs were constitutively expressed in HeLa and Hep3B cells with no significant induction by hypoxia. In contrast, HIF-1α protein was barely detectable in normoxic cells but highly inducible in hypoxic cells, whereas HIF-1β protein was constantly present. Hypoxia-induced HIF-1 binding as well as HIF-1α protein was rapidly and drastically decreased *in vivo* following an abrupt increase to normal oxygen tension. In addition, arrest of protein translation by cycloheximide failed to abrogate HIF-1 DNA binding in hypoxic cells, suggesting that activated HIF-1 is dispensable of protein translation. Moreover, short pre-exposure of cells to hydrogen peroxide selectively prevented hypoxia induced HIF-1 binding via blocking accumulation of HIF-1α protein, while treatment of hypoxic cells or hypoxic cell extracts with H_2O_2 had no effect on HIF-1 binding. Furthermore, removal of H_2O_2 fully restored HIF-1 DNA binding. These results indicate that activation of HIF-1 involves redox dependent stabilization of HIF-1α protein.

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Session 5. FUNCTIONAL RESPONSES TO HYPOXIA (I)

Chairpersons: Peter J. Ratcliffe and Constancio González

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O_2-SENSITIVE ION CHANNELS: FUNCTIONAL ROLES AND MECHANISMS

J. López-Barneo, R. Montoro and J. Ureña
Departamento de Fisiología Médica y Biofísica. Facultad de Medicina, Universidad de Sevilla.

Oxygen tension (PO_2) is known to be an important variable for the regulation of many physiological functions; however, the cellular mechanisms underlying the detection of the changes in PO_2 are not well understood. Chronic exposure to hypoxia can produce the induction of genes encoding for enzymes, hormones and growth factors (1). It is also known that PO_2 regulates the activity of several ion channel types which participate in a number of fast physiological adaptations to hypoxia. O_2-regulated K^+ and/or Ca^{2+} channels have been reported to exist in such diverse locations as glomus cells of the carotid body, systemic and pulmonary arterial smooth muscle, neuroepithelial airway chemoreceptors, mammalian neurons, and pheochromocytoma cells (2). Chemosensory transduction by the carotid body is a well characterized O_2-dependent process. Carotid body (glomus) cells are capable of sensing reductions in arterial O_2 tension (PO_2) and to stimulate the brain stem respiratory centers to produce hyperventilation. Electrophysiological work has shown that the chemoreceptive properties of glomus cells are based upon the presence of O_2-sensitive K^+ channels whose activity is inhibited in response to lowering PO_2 (see for a review ref. 3). Hypoxia results in the enhancement of cellular excitability and leads to Ca^{2+} entry through voltage-dependent channels, transmitter release, and activation of the afferent fibers of the sinus nerve (3,4,5). Monitoring cytosolic $[Ca^{2+}]$ and dopamine release in intact single glomus cells demonstrates a characteristic relationship between PO_2 and transmitter secretion at the cellular level that is comparable to the relation described for the input-output variables in the carotid body (2). Although the interaction of O_2 with the ion channels is not well known, it is highly likely that the underlying mechanisms are delimited to the cell membrane. Reversible, PO_2-dependent changes in channel activity are seen in excised membrane patches and with buffering of intracelular pH and $[Ca^{2+}]$ or after blockade of G-proteins with GTPγS. Changes of PO_2 lead to specific alterations in the kinetic parameters of the channels (deceleration of activation without effects on the open and closed intervals after the first opening as well as on deactivation), thus, it has been proposed that O_2 interacts with specific domains of the channel macromolecule. O_2 could modify channel function via an O_2-sensor associated with the channel macromolecule. As putative O_2 sensor, it has been proposed a membrane-bound NADPH-oxidase (6) which could co-express with

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the channels and, depending on the PO_2 levels, modify the redox status of specific residues in the channel protein. Alternatively, O_2 could bind directly to metal-containing sites located in the main channel subunits and, thus, regulate the conformational state of the molecule. In fact, auxiliary beta subunits in potassium channels are known to have a relatively high degree of sequence similarity with oxidases (7).

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K^+ channels in carotid body type I cells and their sensitivity to hypoxia: studies in chronically hypoxic and developing rats.

Chris Peers

Institute for Cardiovascular Research, Leeds University, Leeds LS2 9JT.

The carotid body mediates increased ventilation in response to reductions in inspired O_2 levels. Such increases in ventilation mature postnatally, such that ventilatory responses in neonates are blunted as compared with adults. Furthermore, in animals born and reared in chronically hypoxic (CH) conditions, ventilatory responses to acutely inspired hypoxic gas mixtures are blunted as compared with age-matched, normoxically reared animals (Hanson & Kumar (1994) *Adv. Exp. Med. Biol.* **360**, 99-108).

Type I cells are believed to be the O_2-sensing elements of the carotid body, and much interest has centred on K^+ channels in these cells which can be reversibly inhibited by hypoxia (see Peers & Buckler (1995) *J. Memb. Biol.* **144**, 1-9). Our studies have utilized type I cells from 10 day old rat pups to indicate that hypoxia inhibits Ca^{2+}-sensitive K^+ (K_{Ca}) channels (e.g. Wyatt & Peers (1995) *J. Physiol.* **483**, 559-565), whilst voltage-gated K^+ channels were unaffected (Peers (1990) *Neurosci. Lett* **119**, 253-256). Carotid bodies from rats of this age are almost fully mature in terms of catecholamine release and carotid sinus nerve discharge in response to hypoxia (Donnelly & Doyle (1994) *J. Physiol.* **475**, 267-275). By contrast, in cells isolated from age-matched rats born and reared in a low Po_2 environment (10% O_2), K_{Ca} channels were lacking. However, acute hypoxia (Po_2 approx. 20mmHg) reversibly inhibited the residual voltage-gated K^+ currents in these CH cells (Wyatt *et al.* (1995) *Proc. Natl. Acad. Sci USA* **92**, 295-299). Thus CH suppressed functional expression of K_{Ca} channels, but hypoxia sensing mechanisms were still present.

We have investigated whether the observed differences between normoxic and CH type I cells arose because CH maintained the carotid body in an immature state. To do this, we compared the properties of normoxically reared 4 day old carotid body type I cells with those isolated from older animals (10 days old and adult). In comparison with cells from older animals, K_{Ca} channels contributed significantly less to total whole-cell K^+ current in 4 day old cells. However, acute hypoxia caused significantly less inhibition of K^+ currents in 4 day olds, and appeared to selectively inhibit K_{Ca} channels in cells from rats of all ages studied. These findings suggest that chronic hypoxia may suppress a postnatal development of K_{Ca} channel functional expression in type I cells, but does not suppress the development of O_2 sensing mechanisms. Additionally, in CH, these sensing mechanisms couple to K^+ channels other than K_{Ca} channels, but in normoxically-reared young animals, where K_{Ca} channels are lacking (as compared with cells from older animals), there is no obvious coupling of O_2-sensing mechanisms to other K^+ channel types.

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OXYGEN SENSING BY RAT CHROMAFFIN CELLS: ADRENAL MEDULLA AND CAROTID BODY CONTRASTED

C.A. Nurse, A. Jackson, R.Thompson and H. Zhong
Department of Biology, McMaster University, Hamilton, Ontario, Canada L8S 4K1

The ability to sense oxygen and make appropriate adjustments for maintaining tissue Po_2 are crucial if mature animals are to survive hypoxia. In mammals, chemoreceptors (type 1 cells) in the carotid body (CB) are the primary O_2 sensors, which regulate blood Po_2 via reflex control of ventilation [Gonzalez et al., 1994; *Physiol. Rev.* 74: 829-898]. In the neonate, however, the ability to survive hypoxic stress depends critically on catecholamine (CA) release from the adrenal medulla and in some species, e.g. rat and man, this occurs prior to the onset of sympathetic innervation [Slotkin & Seidler, 1988; *J. Devel. Physiol.* 10: 1-16]. Since CB type 1 cells and adrenomedullary chromaffin cells (AMC) derive from the embryonic neural crest, and are phenotypically similar, the possibility is raised that they might both express similar O_2 sensing mechanisms.

In whole-cell recordings from neonatal rat AMC, cultured for 1-3 days, the majority (~70%; n= 50) responded to acute hypoxia (Po_2 = ~40 torr) with ~30% suppression of voltage-dependent K^+ current, similar to CB type 1 cells. In addition, hypoxia depolarized neonatal AMC by 10 - 15 mV, in association with a conductance decrease; the depolarization was often sufficient to trigger action potentials. Exposure of neonatal AMC cultures to moderate (10% O_2) or severe (5% O_2) hypoxia for 1 hr caused a dose-dependent, nifedipine-sensitive, stimulation (~3x or 6x respectively) of epinephrine release, determined by HPLC. As expected, hypoxia also stimulated CA release (mainly dopamine) in cultures of neonatal CB type 1 cells.

In contrast to these results on *neonates*, cells isolated from *juvenile* (P13 - P20) rats displayed striking differences. Whereas juvenile CB type 1 cells continued to respond to hypoxia (10% O_2), as revealed by a ~3x stimulation of CA secretion, juvenile AMC were unresponsive during whole-cell recordings and in assays for CA release. It is unknown whether this failure to respond is due to downregulation of particular K^+ channel subtypes and/or the O_2 sensor protein. Thus AMC and CB type 1 cells appear to have similar O_2-sensing mechanisms, though the former present an interesting model where O_2 sensing appears to be regulated by normal developmental processes (e.g. sympathetic innervation). It remains to be determined whether similar K^+ channel subtypes are utilized by both cell types for sensing O_2.

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Role of potassium channels in hypoxic chemoreception in carotid body type-1 cells.

K.J. Buckler
University Laboratory of Physiology, Parks Road, Oxford, OX1 3PT

It has been proposed that the modulation of potassium channel activity by hypoxia plays a major role in oxygen sensing by the type-I cells of the carotid body. To date, two main types of oxygen-sensitive K- channels have been characterised in type-I cells. These are a high conductance (190pS) calcium activated potassium channel (BKca) found in rat type-I cells (Wyatt & Peers 1995), and lower conductance (40pS) calcium insensitive channel (termed the KO2-channel) found in rabbit type-I cells (Ganfornina & Lopez-Barneo 1991). It has been suggested that inhibition of these channels by hypoxia is responsible for membrane depolarisation which in turn induces voltage-gated calcium entry and neurosecretion.

This model of oxygen chemoreception has been called into question by the observation that pharmacological compounds which also inhibit these oxygen sensitive K+-channels e.g. (TEA, 4-AP and charybdotoxin) fail to excite the intact carotid body under normoxic conditions (Donnelly 1995, Pepper et.. al 1995). This paper presents the results of experiments designed to test the role of BKCa and KO2-channels in setting the resting potential of neonatal rat type-I cells and examines the cause of the depolarisation seen in hypoxia.

The effects of several K-channel inhibitors were tested on cells which responded to either hypoxia or anoxia with a large, rapid, rise in [Ca2+]i. These included 20 nM charybdotoxin, 10 mM TEA and 5 mM 4-AP an inhibitor of KO2 and other K-channels. None of these inhibitors evoked a significant rise in [Ca2+]i. Similarly, a combination of both 10 mM TEA & 5 mM 4-AP failed to depolarise type-I cells. Thus neither BKCa -channels nor any other TEA or 4-AP sensitive K+-channels make a significant contribution to the control of the resting membrane potential under normoxic conditions in these cells. Consequently inhibition of any such channels cannot account for the depolarisation seen in hypoxia.

It has been reported, previously, that in cells voltage clamped close to their normal resting potential, anoxia evokes a small inward shift in holding current of a few pA (Buckler & Vaughan-Jones 1994). Anoxia also dramatically reduces resting membrane conductance (measured using voltage ramps from -90 to -30 mV). The I/V relation of this oxygen-sensitive current has a reversal potential of about -90 mV in normal extracellular [K +]o or -60 mV in 20 mM extracellular K+. These results confirm that this oxygen-sensitive current is also carried by K+ ions. This current however was insensitive to both TEA (10 mM) and 4-AP (5 mM) and showed little voltage sensitivity over a wide range of potentials (-100 to +30).

In summary, these investigations have shed new light on the role of K-channels in O2-chemoreception in that it is now clear that the response to hypoxia cannot be explained by the inhibition of large voltage-gated K+-conductances alone. Instead it is proposed that the initial depolarisation, or receptor potential, is mediated via the inhibition of a small resting K+--conductance. This will probably serve to initiate electrical activity and Ca2+-influx, which could then be further controlled by the modulation of voltage-gated/Ca2+-dependent K+-channels.

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Session 6. FUNCTIONAL RESPONSES TO HYPOXIA (II)

Chairpersons: María F. Czyzyk-Krzeska and Chris Peers

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Oxygen Sensing in the Pulmonary Vasculature

E. Kenneth Weir, Helen L. Reeve, Simona Tolarova
David N. Cornfield, Daniel P. Nelson, Stephen L. Archer

In the smooth muscle cells of resistance arteries the resting membrane potential is largely determined by potassium current. The effect of changes in oxygen tension on membrane potential and vascular tone appears to be mediated through changes in potassium channel gating. In the case of the resistance pulmonary arteries of the rat, hypoxia inhibits one or more delayed rectifier (K_{DR}) channels, causes membrane depolarization and calcium entry through voltage-dependent calcium channels, thus initiating vasoconstriction (1). The mechanism by which the gating of the K_{DR} channel changes in response to hypoxia is not clear, but might involve alteration of redox status (2,3). It has been suggested that NADPH oxidase might be the oxygen sensor (4). However, transgenic mice which lack the 91 kD subunit of NADPH oxidase (5), still have normal hypoxic pulmonary vasoconstriction, which makes a role for NADPH oxidase unlikely. Despite this, Shaker K^+ channel beta subunits belong to an NAD(P)H oxidoreductase superfamily (6), which again raises the question of a redox gating mechanism.

The means by which oxygen changes K^+ channel gating is made more intriguing by the observation that an increase in oxygen tension inhibits, rather than activates, a K_{DR} channel in the smooth muscle of the ductus arterious (7). The inhibition causes membrane depolarization and initiates normoxic constriction of the ductus at birth. In other species and in other vessels K_{Ca} channels in the smooth muscle cells also respond differently to changes in oxygen tension. Hypoxia inhibits K_{Ca} channels in the resistance pulmonary arteries of the fetal lamb (8), while hypoxia activates K_{Ca} channels in the cerebral arteries of the cat (9). These observations imply either different oxygen-sensing components in the K^+ channels, or different cellular oxygen metabolism giving rise to diametrically opposite effects on fundamentally the same channels. The problem of the mechanism of K^+ channel gating also arises in studies on oxygen-sensing in the carotid body (10) and the neuroepithelial body (11).

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Mechanisms of Hypoxic Pulmonary Vasoconstriction: The Role of O_2-sensitive Voltage-gated K^+ Channels

Xiao-Jian Yuan

Department of Medicine,

Division of Pulmonary and Critical Care Medicine;

Department of Physiology,

University of Maryland School of Medicine,

Baltimore, Maryland 21201

Running title: Hypoxic Regulation of K^+ Channel

Address correspondence to:

Xiao-Jian Yuan, M.D., Ph.D.

Assistant Professor of Medicine and Physiology

Division of Pulmonary and Critical Care Medicine

University of Maryland School of Medicine

10 S. Pine St., Suite 800, Baltimore, MD 21201, U.S.A.

Tel: (410)706-8103; Fax: (410)706-8162

E-mail: xyuan@umabnet.ab.umd.edu

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A pivotal procedure in hypoxic pulmonary vasoconstriction is the increase in cytoplasmic free calcium concentration ($[Ca^{2+}]_i$) in pulmonary arterial smooth muscle cells.[1, 2] $[Ca^{2+}]_i$ is controlled by Ca^{2+} influx through sarcolemmal Ca^{2+} channels[3] and Ca^{2+} release from intracellular stores.[2] Membrane potential (E_m) regulates $[Ca^{2+}]_i$ in large part through the voltage-dependence of Ca^{2+} channels.[3] The activity of voltage-gated K^+ (K_V) channels is a major determinant of resting E_m,[4] while hypoxia selectively inhibits K_V channels in pulmonary arterial smooth muscle cells.[5] Thus, the reduced K_V currents ($I_{K(V)}$) depolarizes the cells, opens voltage-gated Ca^{2+} channels, increases $[Ca^{2+}]_i$ and induces pulmonary vasoconstriction.[4,5] The rise in $[Ca^{2+}]_i$ also activates Ca^{2+}-activated Cl^- channels to further depolarize the cells and activates Ca^{2+}-activated K^+ channels to repolarize the cells. Hypoxia-induced inhibition of Ca^{2+}-activated K^+ channels helps to maintain the depolarization and thus the pulmonary vascular tone.[6]

Hypoxia decreases ATP generation[7] (via decreased citric acid circle and oxidative phosphorylation), alters cellular redox potential,[8] reduces cytochrome P-450 activity[9,10] and diminishes oxidant production.[8] Interestingly, $I_{K(V)}$ is also decreased by the metabolic inhibitors (deoxyglucose and FCCP),[11,12] reducing agents (glutathione)[11,13] and the P-450 inhibitors (clotrimazole and 1-aminobenzotriazole),[10] while is increased by ATP[14] and oxidants (diamide and nitric oxide).[15] Molecularly, K_V channels are composed of pore-forming α-subunits and auxiliary ß-subunits ($\alpha_4\beta_4$).[13] At least five K_V channel α-subunit genes and three ß-subunit genes are expressed in pulmonary arterial smooth muscle cells. The recent finding that ß-subunits belong to an NAD(P)H oxidoreductase superfamily[16] suggests that the ß-subunits may serve as an intermediate in sensing O_2 tension and redox status change to govern the native K_V channel functions.

The data mentioned above suggest that the mechanisms by which hypoxia decreases $I_{K(V)}$ may be related to: **a)** inhibition of cellular metabolism, **b)** change in redox potential, **c)** inhibition of the activity of cytochrome P-450 or NADPH reductases (or a membrane-delimited, metal-containing protein) and/or **d)** conformational change of the channel protein.

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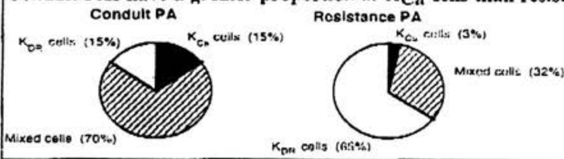
Longitudinal Differences in K_{Ca} and K_{DR} channel distribution determine vascular reactivity to hypoxia Stephen L. Archer, James Huang, Helen Reeves, E. Kenneth Weir
Veterans Affairs Medical Center and University of Minnesota, Minneapolis, MN, USA

Hypoxic pulmonary vasoconstriction (HPV) is defined as the rapid, reversible, increase in pulmonary vascular resistance which occurs in the lung, or a segment of lung, beginning within seconds of a decrease in alveolar O_2 tension below a threshold (~FiO_2 0.1). The role of HPV in the adult is the regulation of ventilation/perfusion matching. HPV is intrinsic to the smooth muscle cell (SMC)[1,2], although the magnitude of the response is modulated by circulating mediators (e.g. endothelin). Hypoxic constriction is unique to the pulmonary circulation, most systemic vessels dilate in response to hypoxia [3]. Hypoxia increases free cytosolic calcium concentration $[Ca^{2+}]$, and causes constriction of pulmonary artery (PA), but not cerebral artery, SMCs[1,2]. This implies there is a different O_2 sensing mechanism in the pulmonary vasculature. Even within the pulmonary circulation the vessels primarily responsible for regulating tone (small, muscular, resistance arteries) display a sustained constriction to hypoxia whereas the larger, proximal, conduit pulmonary arteries exhibit a transient constriction followed by a prolonged dilation, much like a systemic artery[3]. There is good evidence that HPV results from the integrated action of Ca^{2+} and K+ channels in the plasmalemma [4]. The Ca^{2+} channel is crucial in providing the Ca^{2+} for contraction, but they are primarily regulated by the activity of K+ channels, which control membrane potential (E_M)[5]. A major advance in our understanding of HPV is the recognition that hypoxia-induced membrane depolarization results from inhibition of K+ channels in PA SMC. Thus far, candidate O_2-inhibited K+ channels have been identified via patch clamp techniques based on their conductance and sensitivity to pharmacological inhibitors. PA SMC cells contain various types of K channels including: calcium-sensitive (K_{Ca}[5]), delayed rectifier (K_{DR} [6]) and adenosine triphosphate(K_{ATP})-gated K+ channels[7]. Hypoxia inhibits a family of 4-aminopyridine (4-AP)-sensitive, voltage-gated (Kv), K+ channels. There is cell-cell and segment-segment variation in the predominant K+ channel types expressed by PA SMCs (Figure).

Conduit PAs have a greater proportion of K_{Ca} cells than resistance PAs [8]



In some cells, whole cell K+ current (I_K), in the resting condition, is carried mainly by K_{DR} channels, in others by K_{Ca} channels and in others by both channel types. This lead us to name these cell "types" K_{DR}, K_{Ca} and Mixed". The following criteria differentiate the 3 cell types: pharmacology (sensitivity of I_K to tetraethylammonium, TEA vs 4-AP), biophysical attributes (current density, capacitance) and cell morphology (light microscopy). K_{Ca} cells are large, smooth, have low current density and are TEA-sensitive. In contrast, K_{DR} cells are small, have a perinuclear bulge, high current density and are 4-AP-sensitive [8]. The prevalence of K_{Ca} and K_{DR} cells varies between conduit (main or first division PAs) and resistance (4th-5th division, <400 µm, intraparenchymal PAs) arteries. K_{Ca} cells are more common in the conduit and K_{DR} cells are more common in resistance PAs (Figure). Though a cell's I_K may be predominated by 1 channel type, each cell contains several channel types. The I_K is a mosaic, derived from all active K+ channels. The occurrence of diverse electrophysiologic cell types within and between vascular segments contributes to regional differences in vascular reactivity to stimuli which influence tone via K+ channels. The functional importance of K+ channel diversity is evident in studies of segmental differences in the response of the PA to stimuli which alter tone due to effects on K+ channels. For example, hypoxia inhibits K_{DR} channels while activating K_{Ca} channels [8]. Hypoxia constricts resistance PA rings (rich in K_{DR} cells) while relaxing conduit rings (rich in K_{Ca} cells) [8]. We conclude that regional diversity in the distribution of O_2-sensitive K+ channels contributes to the regional localization of HPV to resistance PA.

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CALCIUM CHANNELS, CYTOSOLIC CALCIUM AND VASOMOTOR RESPONSES TO HYPOXIA.

A. Franco-Obregón*, J. Ureña, T. Smani, J. López-Barneo
Departamento de Fisiología Médica y Biofísica. Facultad de Medicina, Universidad de Sevilla. *Present address: Department of Pharmacology, Mayo Clinic, Rochester, MN, USA.

The regional distribution of blood in most vascular beds is determined by local variations in oxygen tension. As is characteristic of most systemic vascular beds, the main trunk and larger branches of pulmonary artery vasodilate in response to hypoxia. The opposite response, or hypoxic vasoconstriction, is observed for the more distally located resistance branches of the pulmonary artery (1-3). Several cellular mechanisms have been proposed to explain the distinct vasomotor responses to hypoxia of the systemic-like and distal pulmonary arterial segments. Some models implicate the role of vasoactive agents (e.g. NO and endothelin 1) released from the endothelium in response to regional variations in blood oxygen content. These endothelium-derived vasoactive factors then diffuse to the arterial smooth muscle layer where they ultimately influence smooth muscle contraction. However, hypoxic vasomotor responses can be partially recapitulated in arterial samples denuded of the endothelium as well as in isolated arterial smooth muscle cells, suggesting that the endothelium plays a modulatory, rather than obligatory, role in the manifestations of the hypoxic-vasomotor responses. Other models propose that the contractile status of arterial smooth muscle is directly responsive to blood PO_2. It seems likely that both endothelium-and smooth-dependent mechanisms work in coordination to elaborate the intact hypoxic-vasomotor responses. In addition, any mechanism(s) responsible for setting arterial tone, regardless of whether dependent on endothelium-derived vasoactive factors or inherent to the smooth muscle cell itself must first impinge on the modulation of vascular smooth muscle cytosolic calcium concentration ($[Ca^{2+}]_i$) which then ultimately determines smooth muscle contraction (1-3).

To investigate what aspect of these responses are inherent to the smooth muscle cell of arteries we examined acutely dissociated arterial smooth muscle cells from various arterial beds and monitored their responses to changes in bathing PO_2 under different experimental paradigms. Using calcium microfluorimetry, Fura-2 AM loaded vascular smooth muscle cells isolated from various systemic and pulmonary arteries exhibit oscillations of cytosolic calcium, or Ca^{2+} spikes. The frequency and amplitude of the Ca^{2+} spikes, as well as basal $[Ca^{2+}]_i$, were exquisitely sensitive to changes bathing PO_2. Most characteristically, basal $[Ca^{2+}]_i$ was

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lowered in both systemic and the majority of main trunk pulmonary myocytes following a reduction in PO_2. By contrast, basal $[Ca^{2+}]_i$ was often augmented in myocytes isolated from the distal branched of the pulmonary artery (4). Since in the same preparation basal $[Ca^{2+}]_i$ (as well as Ca^{2+} spike frequency and amplitude) are similarly regulated by Ca^{2+} influx (4), we next examined the possibility that the primary site of action of oxygen tension was on voltage gated Ca^{2+} channels as recorded using the whole-cell patch clamp technique. In agreement with our Fura-2 measurements the macroscopic L-type Ca^{2+} currents of systemic and main pulmonary myocytes were attenuated in response to a drop in PO_2 at negative membrane potentials near the physiological resting potential of vascular smooth muscle (5,6). Further corroborating our previous results we found that the L-type Ca^{2+} currents of myocytes isolated from the distal branches of pulmonary artery were potentiated by hypoxia near rest (6). It thus appears that the O_2-modulation of L-type Ca^{2+} channels influences basal $[Ca^{2+}]_i$ in a manner which is consistent with the classically described vasomotor responses of systemic and distal pulmonary arterial beds to hypoxia.

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Title: Hypoxia, adenosine, and K_{ATP} channels of coronary arterial smooth muscle.

N.B. Standen, Ion Channel Group, Department of Cell Physiology and Pharmacology, University of Leicester, Leicester LE1 9HN, UK.

The delivery of oxygen to tissues is matched to their local needs by changes in blood flow, and hypoxia causes vasodilation on many vascular beds. In the heart, for example, a local fall in oxygen tension causes dilation of coronary arteries and arterioles, increasing blood flow and oxygen supply. The vasodilation results from relaxation of coronary arterial smooth muscle, and could occur by several means: an action of O_2 tension on the smooth muscle cells either directly or by changing the concentration of intracellular metabolites, or release of vasodilator factors from either endothelial cells or cardiac myocytes. Adenosine released from cardiac myocytes has been proposed to play a central role in matching blood supply through the coronary circulation to the needs of the myocardium.

A number of studies either in whole animals or isolated hearts have suggested that ATP-sensitive K^+ channels (K_{ATP} channels) may provide an important mechanism for hypoxic coronary vasodilation, since such vasodilation may be substantially inhibited by the sulphonylurea K_{ATP} channel blocker, glibenclamide [1,2]. Part of the vasodilation appears to depend on adenosine release, while part is independent of adenosine. Adenosine has also been shown to hyperpolarize coronary arterial smooth muscle cells, consistent with the activation of K^+ channels.

In smooth muscle cells isolated enzymatically from several different vascular tissues, inhibition of metabolism by inhibiting either glycolysis or oxidative metabolism or both leads to the activation of glibenclamide-sensitive K^+ currents [3]. In pig coronary arterial smooth muscle cells studied using perforated patch recording, adenosine activated K^+ currents that showed little voltage dependence and had a pharmacology consistent with the activation of K_{ATP} channels [4]. The underlying single channels had a conductance of 35 pS in high $[K^+]$ solution. The action of adenosine was mimicked by an A_1 adenosine agonist (CCPA) and inhibited by the A_1 antagonist DPCPX, but was unaffected by A_2 agents. In rabbit mesenteric artery, however, there is evidence that adenosine can activate K_{ATP} channels by way of A_2 receptors activating the adenylyl cyclase-PKA pathway [5]. In pig coronary cells, we have also found that superfusion of a solution with a reduced oxygen tension (pO_2 25-40 mmHg) caused activation of K^+ currents [6]. The currents showed the same lack of voltage-dependence and sensitivity to glibenclamide as adenosine-activated currents in the same preparation. The single channel conductance, measured either under whole-cell recording conditions or in cell-attached patches, was also identical to that of adenosine-activated channels. It therefore appears that hypoxia can activate K_{ATP} channels in these cells, though the mechanism remains unclear, since such pO_2 levels would be expected to cause little change in intracellular nucleotide concentrations.

Thus activation of K_{ATP} channels in arterial smooth muscle provides one mechanism for hypoxic vasodilation in the coronary circulation, and probably in a number of other vascular beds. K^+ channel activation will lead to hyperpolarization of smooth muscle cells, causing relaxation by reducing entry and possible release of Ca^{2+}. In addition to their physiological control, K_{ATP} channels can be activated by a number of synthetic K^+ channel openers. Recent evidence suggests that these compounds may be more potent under conditions of hypoxia or metabolic compromise.

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Session 7. O_2 - SENSING IN NERVE CELLS

Chairperson: Olaf Pongs

Instituto Juan March (Madrid)

ION CHANNEL FUNCTION DURING HYPOXIA: DIRECT AND INDIRECT MODULATION

Gabriel G. Haddad, M.D.

Depending on its severity and duration, O_2 deprivation activates mechanisms that can lead to profound deleterious changes in neuronal structure and function or to adaptive mechanisms that can possibly delay injury and increase neuronal survival. One of these neuronal adaptive mechanisms is believed to be the activation of K^+ channels, but direct evidence for their activation is lacking. We performed experiments to test the hypothesis that hypoxia induces activation of K^+ channels via changes in cytosolic and membrane factors. In cell-attached patches from central neurons, hypoxia reversibly activated an outward current. This hypoxia-activated current in excised inside-out patches was K^+ selective and voltage dependent, and had a high sensitivity to internal ATP, ADP, and AMP-PNP. In cell-free excised membrane patches, we also found that this specific K^+ current was reversibly inhibited by lack of O_2. This was characterized by a marked decrease in channel open-state probability and a slight reduction in unitary conductance. The magnitude of channel inhibition by O_2 deprivation was closely dependent on O_2 tension. The PO_2 level for 50% channel inhibition was about 10 mmHg with little or no inhibition at $PO_2 \geq 20$ mmHg. These results therefore provide the first evidence for regulation of K^+ channel activity by O_2 deprivation in cell-free excised patches from central neurons.

The voltage-sensitive Na channels are another example of a channel that is modulated by low O_2 in nerve cells. The effects of brief exposures to hypoxia on the membrane currents of isolated hippocampal CA1 neurons were studied with the use of the whole-cell variation of the patch-clamp technique. Neurons were acutely dissociated from immature and mature rats. With hypoxia, there was a decrease in the magnitude of the hyperpolarizing holding current and a depression of the voltage-dependent inward current of mature neurons. The hypoxia-sensitive inward current was blocked by tetrodotoxin (TTX) but was not blocked by cadmium or cesium + tetraethylammonium (TEA). Therefore this current was identified as the voltage-dependent, fast-inactivating sodium current (I_{Na}). In mature neurons, hypoxia left-shifted the steady-state inactivation curve for I_{Na}. Whole-cell voltage-clamp studies of acutely isolated human neocortical pyramidal neurons demonstrate that anoxia and metabolic inhibition produce a large negative shift in the steady-state inactivation curve for the voltage-dependent sodium current (I_{Na}). Brief exposures to hypoxia alter therefore the intrinsic excitability of CA1 neurons by at least two mechanisms: 1) alterations in leakage currents and 2) alterations in the fast Na^+ conductance. We propose that the alterations in both K^+ and Na^+ conductances may play an adaptive role by reducing O_2 demands and thus possibly delaying neuronal injury.

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Ischemia and Oxidative Regulation of Neuronal Ca^{2+}-Permeable AMPA Receptors

Dugan, L.L., Turetsky, D.M., Sensi, S.L., Canzoniero, L.M.T. and Choi D.W.

The majority of AMPA-type glutamate receptors are relatively impermeable to Ca^{2+} However, recent studies have identified subpopulations of neurons in hippocampus, cerebellum, spinal cord and cortex which are highly permeable to Ca^{2+}; calcium-permeability may be conferred on AMPA receptors by absence of a GluR-B subunit, the subunit which normally limits Ca^{2+} entry through the AMPA channel. Neurons bearing Ca^{2+}-permeable AMPA receptors show enhanced susceptibility to the glutamate receptor agonist, kainate, and appear to be selectively vulnerable to ischemic injury. In addition, Pelligrini-Giampietro et al (1993) reported a preferential reduction in Glur-B mRNA in hippocampal neurons following transient forebrain ischemia, suggesting that events associated with ischemia or reperfusion may regulate GluR-B expression and increase Ca^{2+}-permeability of AMPA receptors.

Several groups have reported rapid production of reactive oxygen species during brain ischemia-reperfusion . Oxygen radicals during reperfusion may be generated by neurons undergoing glutamate receptor-mediated excitotoxicity, or from damaged mitochondria re-exposed to oxygen. Reactive oxygen species may, in turn, affect redox-sensitive enzymes, transporters, receptors and transcription factors. We hypothesized that reactive oxygen species could change the expression of Ca^{2+}-permeable AMPA receptors.

Neurons with Ca^{2+}-permeable AMPA receptors can be identified by a histochemical stain for kainate-activated cobalt uptake, or by fura-2 microfluorimetry. Brief sublethal exposure to H_2O_2 (60 µM, 30 min), to generate hydroxyl radical, produced a 25% increase in the number of cortical neurons exhibiting kainate-activated cobalt uptake 12-24 hr later. Superoxide anion (generated by xanthine / xanthine oxidase / catalase) or nitric oxide failed to affect the number of neurons with Ca^{2+}-permeable AMPA receptors. Increased kainate-activated cobalt staining remained sensitive to the AMPA/kainate receptor antagonist, 6,7-dintroquinoxaline-2,3-dione (NBQX), and was associated with kainate-stimulated increases in intracellular Ca^{2+} and Ca^{2+} uptake. This increase was accompanied by a decrease in the amount of newly-synthesized neuronal GluR-B, detected by ^{35}S-Met metabolic labeling and immunoprecipitation of GluR protein. These data suggest that oxidative stress can alter the configuration of AMPA receptors on cortical neurons to favor Ca^{2+}-permeable behavior.

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Session 8. SENSITIVITY TO HYPOXIA

Chairperson: Gabriel G. Haddad

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The maturation of peripheral chemoreceptor hypoxic sensitivity.

P.Kumar & D.R.Pepper. Department of Physiology, Medical School, University of Birmingham, Birmingham B15 2TT, UK.

We have been interested in the factors which govern the well-established maturational changes observed in the reflex ventilatory sensitivity to hypoxia that is observed in a number of mammalian species. Our work is focused at electrophysiological events occurring at the carotid body and this poster will describe some of our recent studies.

The large rise in arterial Po_2 that occurs upon the initiation of air breathing at birth in mammals is believed not only to be the reason for a need to increase hypoxic chemosensitivity above *in utero* levels but also to be the cause of this elevated sensitivity. We have characterised this plasticity in the hypoxic sensitivity of the carotid body in animals at various ages after birth and have also shown the 'blunting' effect of preventing the natural elevation in Po_2 by maintaining animals in normobaric hypoxia from birth. More recently we have demonstrated that this blunting effect of chronic hypoxia can be reversed by a return to normoxia and that it is dependent upon the age at which the chronic hypoxia is imposed.

Our data at the level of afferent discharge in the whole organ *in vitro* is now being complemented by studies on isolated type I cells. We have confirmed that chronic hypoxia from birth can reduce the peak outward current of these cells. In collaboration with Dr Chris Peers and colleagues, we have also demonstrated that the K_{ca} component increases with increasing postnatal age and that in very young animals the reduced K_{ca} is associated with a greatly reduced hypoxia sensitivity. Whether this correlation between the afferent chemoreceptor discharge response to hypoxia and K_{ca} channel density are causally related remains to be determined. However, in support of a role for K_{ca} in hypoxia sensing by the carotid body we have shown that 100nM charybdotoxin can increase chemoreceptor discharge in adult animals albeit at relatively hypoxic levels. The effect of this toxin in the neonatal response to hypoxia is currently under investigation.

This work is supported by The Lister Institute for Preventive Medicine, The Medical Research Council and The Wellcome Trust.

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SENSITIVITY TO PHYSIOLOGICAL HYPOXIA.

C. González. Departamento de Bioquímica y Biología Molecular y Fisiología. Facultad de Medicina. Universidad de Valladolid. 47005 VALLADOLID. Spain.

The first topic that I shall address is the definition of physiological hypoxia. Physiological hypoxia is a hypoxic hypoxia produced by ambient factors, i.e., low barometric pressure or high altitude, and proceeding without any pathology for the entire life-span of individuals and from generation to generation. A reasonable limit for physiological hypoxia is an altitude of $\approx$4,000 m above sea level, corresponding to a barometric pressure of 460 mmHg, an inspired PO_2 of 93 mmHg and, before acclimatization, an alveolar PO_2 of $\approx$42 mmHg. Nearly 15 million people lives at this altitude, and when adequate corrections are made for racial and nutritional factors, they exhibit no differences with sea level inhabitants in reproduction, growth or physical performance. Pioneer studies on the physiology of high altitude revealed that and increase in red cell mass and a hyperventilation were the adaptative changes observed in permanent residents at high altitude as well as in lowlanders adapted to high altitude. It was later recognized that many populations of high altitude residents, and all adapted lowlanders, exibited variable degrees of pulmonary hypertension; the adaptative significance of this response has also been documented. A common property of the three cell types involved in the genesis of the adaptative responses, erytropoietin-producing cells of the kidney, chemoreceptor cells of carotid body and smooth muscle cells of the pulmonary artery, is that they possess a threshold to hypoxia which corresponds to an arterial PO_2 of around 70 mmHg, implying that they start to be activated before the arterial O_2 content drops significantly. This low threshold, in turn, means that these cell types should possess O_2-sensors with low affinity to O_2. Another common property of these cell types is that their responses are directed to the entire organism, aiming to prevent hypoxia in bodily cells and to maintain their metabolic rate and function. A corollary follows from these considerations, namely, that in order to prevent hypoxia from increasing in the organism, these three cell types must be extremely active during hypoxia. All these properties define, in my opinion, physiological hypoxia and physiological sensitivity to hypoxia (González et al., 1995).

It should be noted, however, that all cell types in the organism exhibit a general sensitivity to hypoxia, if it is intense enough. The responses observed in most cells are directed to the own survival of the cells, and include shifts in metabolic routes, induction of glycolitic and oxidative enzymes, a general reduction of biosynthetic functions, and at the same time increase in the rate of synthesis of stress proteins acting as molecular chaperons or helping to repair possible mutations induced by hypoxia. These general responses of the cells would mimic those encountered in lower organisms (Bunn and Poyton 1996).

My contention is that even if the O_2-sensor for the general sensitivity to hypoxia and for the physiological hypoxia belongs chemically to the same family, probably a hemoprotein, the sensor for physiological hypoxia has had to acquire some properties through evolution that enables it to detect the adequate levels of hypoxia. Parallel adquisition of specific transduction cascades in the cells endowed with physiological sensitivity to hypoxia would allow them to generate appropriate responses and maintain a high metabolic rate during hypoxia.

In my talk I would make some considerations on the significance of mitochondria and NAD(P)H in the sensing of physiological hypoxia.

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POSTERS

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Regulation of rat liver SAM-synthetase by hypoxia.

Matías A. Avila, Elías N. Rodríguez, Isabel Varela-Nieto, José M. Mato.
Instituto de Investigaciones Biomédicas. CSIC. Arturo Duperier 4, 28029, Madrid, España

S-adenosylmethionine(SAM)-synthetase catalyses the first step of liver methionine metabolism. The formation of SAM involves the transfer of the adenosyl moiety of ATP to methionine, and is the preferred pathway for methionine breakdown in mammals. SAM serves as the methyl donor for essentially all known biological methylation reactions in while it is converted to S-adenosylhomocysteine(SAH), and participates in the synthesis of polyamines and gluthatione. Several liver disorders, such as alcoholic cirrhosis, have been associated with an impairment of methionine metabolism and the methylation cycle. Low oxygen availability is a condition associated with alcoholic liver cirrhosis and also occurs during liver transplantation. In a model of kept rat hepatocytes in primary culture, we have studied the regulation of SAM-synthetase during hypoxia (3% O_2). Our results show a time-dependent reduction of enzyme activity along with a decreased value of the ratio SAM/SAH. SAM synthetase steady-state mRNA levels were downregulated rapidly after the onset of hypoxia (2 h), suggesting an effect on mRNA stability rather than a change in the transcription rate, given the long half-life of SAM synthetase mRNA (> 8 h). Our observations indicate that liver SAM synthetase activity and expression are acutely regulated during hypoxia. To our knowledge this is the first example of a gene being downregulated through a post-transcriptional mechanism by hypoxia.

Instituto Juan March (Madrid)

University Laboratory of Physiology
Parks Road, Oxford OX1 3PT, UK

Switchboard: +44-1865-272500
Fax: +44-1865-272469

KEITH J. BUCKLER

Mechanisms of O_2-sensing.

Although hypoxia inhibits a number of different ion-channels in isolated type-I cells the sensor responsible for detecting changes in O_2-concentration is unknown. I am investigating a number of possible O_2-sensing pathways. At present research is directed at two mechanisms which have achieved a degree of prominence in the literature, these are 1) that O_2-chemoreception is linked to oxidative phosphorylation or 2) that O_2-chemoreception is mediated through changes in H_2O_2 production via the action of an NAD(P)H oxidase.

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Swelling-, cAMP- and acidosis-induced chloride currents recorded from isolated rat carotid body type I cells

E. Carpenter and C. Peers

Institute for Cardiovascular Research, University of Leeds, Leeds LS2 9JT, UK

It has been proposed that the carotid body can act as an 'osmoreceptor' [1,2]. Many chloride channels are known to be activated by hypotonic solutions [3] and are often activated by cyclic AMP [3], the levels of which rise in carotid body type I cells during hypoxia [4]. Regulation of chloride channels by pH has also been reported [5]. We therefore decided to investigate if such channels were present in rat carotid body type I cells.

We have used the whole cell patch clamp technique to record chloride currents from type I cells isolated from 10 day old rats as previously described [6]. The extracellular solution contained (in mM): 67.5 NaCl, 48.3 TEA-Cl, 5 KCl, 6 $MgCl_2$, 0.1 $CaCl_2$, 5 HEPES, 10 D-glucose (pH 7.40 w/NaOH or TEA-OH in Na^+-free solutions, 300mOsm w/sucrose, 21-24°C). Hypotonic solutions were typically 240mOsm, although some experiments were carried out using 250-260mOsm. Symmetrical Cl^- was used and pipettes had a resistance of 4-9MΩ when filled with (in mM): 10 NaCl, 117 CsCl, 1 $CaCl_2$, 2 $MgCl_2$, 11 EGTA, 11 HEPES, 2 Na_2ATP (pH 7.20 w/CsOH, 300mOsm w/sucrose, 21-24°C). Cells were held at 0mV and current-voltage (I-V) relationships were obtained using either voltage steps (50ms, 0.2Hz) or ramps (400ms, 0.2Hz).

A 3-4min hypotonic challenge induced an outwardly rectifying, non-inactivating conductance in 9 cells tested (-132 ± 32pA at -80mV, 262 ± 66pA at +80mV). Cells could be seen to swell under the microscope during the hypotonic challenge. On return to isotonic conditions current levels reduced close to control levels. Using voltage ramps the cells were challenged with the hypotonic solution until a steady-state current was obtained, usually within 3-7min (-312 ± 40pA at -80mV, 522 ± 58pA at +80mV, n = 26). The reversal potential was 1.6 ± 0.6mV (n = 26), close to the theoretical reversal potential of 0mV under these conditions. Replacing extracellular Na^+ with choline$^+$ (n = 6) did not significantly affect the reversal potential or current amplitude, indicating that it is unlikely that this current arose from a non-specific cationic channel. When the extracellular Cl^- concentration was reduced by replacing NaCl with Na-gluconate the reversal potential was shifted by 14.7 ± 0.4mV (n = 5) in the positive direction, close to the theoretical shift of 17.8mV, suggesting that the current was carried by Cl^-. Directly removing NaCl produced a similar shift in reversal potential (n = 3). The current was sensitive to blockade by niflumic acid (300μM, 81.7 ± 4.8% inhibition at -80mV, 76.3 ± 5.7% at +80mV, n = 5) and the stilbene derivative DIDS although the effect of DIDS was predominantly on the outward current (200μM, 15.5 ± 4.9% inhibition at -80mV, 62.8 ± 1.6% at +80mV, n = 6). A niflumic acid-sensitive outwardly rectifying conductance with a reversal potential of 2.3 ± 2.1mV was also induced by isotonically applied 8-Br-cAMP (2mM, -30 ± 6pA at -60mV, 57 ± 13pA at +60mV, n = 5). Intracellular acidosis effected by isotonically applied Na-propionate (20mM) also induced an outwardly rectifying conductance with a reversal potential of -0.5 ± 0.9mV (-70 ± 21pA at -60mV, 104 ± 35pA at +60mV, n = 5).

These data support the suggestion that the carotid body might play a role as an 'osmoreceptor'. More importantly, the Cl^- conductance reported here may be involved in the overall response of type I cells to physiological stimuli.

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SELECTIVE INHIBITION OF A SLOW-INACTIVATING VOLTAGE-DEPENDENT POTASSIUM CHANNEL IN PC12 CELLS BY HYPOXIA

Laura Conforti and David E. Millhorn
Department of Molecular and Cellular Physiology, College of Medicine, University of Cincinnati, Cincinnati, OH 45267-0576, USA

The pheochromocytoma (PC12) cell line consists of an homogenous population of oxygen-sensitive cells which can be used as a model system to study the modulation of ion channels and gene expression by hypoxia. We recently discovered that hypoxia inhibits an O_2-sensitive K current in PC12 cells which causes depolarization followed by an increase in intracellular calcium and secretion of dopamine (Zhu et al., Am. J. Physiol. 271: C658, 1996). In order to characterize the O_2-sensitive K channel, the effect of hypoxia on the different K channels present in PC12 cells was studied at the single-channel level in cell-attached and inside-out configurations. Ensemble-average current, conductance and open probability were measured before and after exposure to hypoxia (10% O_2, 80 mmHg Po_2 measured in the perfusion chamber). Experiments were performed at room temperature with an extracellular concentration of K^+ of 2.8 mM. Ramp pulse depolarization experiments showed that hypoxia inhibits a 20 pS K channel (66 $\pm$ 12% inhibition). Four types of K channels were recorded in PC12 cells: a small conductance channel (14 pS), a calcium-activated channel (150 pS) and two K channels with similar conductance (20 pS). These last two channels differ in their time-dependent inactivation (measured with step pulse depolarization from an holding potential of -60 mV to +50 mV, 200 ms duration): one is a slow-inactivating channel, while the other belongs to the family of fast transient K channels. Of these, only the slow-inactivating 20 pS K channel is sensitive to hypoxia. Exposure to 10% O_2 produced a reduction in ensemble current amplitude and a decrease in open probability (NxPo ca. 50%) with no change in either single-channel conductance or number of active channels in the patch. This response, when studied in inside-out patches, was reversible upon returning to normoxia. In order to identify the molecular nature of the O_2-sensitive K channel we studied the expression of genes that encode for the α subunits of the delayed rectifier type of K channel in PC12 cells and their regulation upon prolonged exposure to hypoxia. Reverse-transcriptase polymerase chain reaction (RT-PCR) experiments showed that at least two genes of the *Shaker* family (Kv1.2 and Kv1.3) are expressed in this cell line together with Kv2.1, Kv3.1 and Kv3.2. The expression of the *Shaker* Kv1.2, but none of the other K channel genes, increased in cells exposed to 10% O_2 for 18 hr. Kv1.2 is known to encode for slow-inactivating outward K channels of similar conductance to the O_2-sensitive K channel which we recorded in PC12 cells. In conclusion, the O_2-sensitive K channel in PC12 cells is a 20 pS slow-inactivating K channel. This channel appears to belong to the *Shaker* subfamily of voltage-gated K channels.

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Arachidonic Acid does not mimic the effects of hypoxia on isolated rat carotid body type I cells.

C. J. Hatton and C. Peers

Institute for Cardiovascular Research, University of Leeds, Leeds LS2 9JT, UK

Hypoxic inhibition of K^+ currents in type I cells is believed to be obligatory for chemoreception in this organ [1]. We have previously demonstrated that inhibition of cytochrome P-450 can prevent the hypoxic inhibition of K^+ currents in these cells [2]. Here, we have investigated the effects of arachidonic acid, a major substrate for cytochrome P-450 on the ionic currents of isolated carotid body type I cells of the rat.

Cells were isolated and maintained as previously described [2], and whole-cell currents were recorded using the patch clamp technique (holding potential -70mV). K^+ or Ba^{2+} currents were evoked by 50ms depolarising pulses (0.2Hz) to +20mV (for K^+ currents), or to 0mV (for Ba^{2+} currents), or to a range of test potentials (-60 to +60mV) to construct current-voltage relationships. Drug effects are expressed as percentage (±s.e.m.) change from control amplitudes.

Arachidonic acid (2μM-20μM) inhibited K^+ currents in a concentration dependent manner (up to 88.8±3.1%), at all activating potentials studied. In high (6mM) Mg^{2+}, low (0.1mM) Ca^{2+} perfusate (which inhibits the Ca^{2+} activated component of the whole-cell K^+ current [3]) arachidonic acid 2μM still inhibited K^+ currents, although this was significantly less than in control solutions, suggesting some selectivity for the hypoxically sensitive calcium-activated component of the K^+ current. However, 2μM arachidonic acid also significantly inhibited the whole cell Ba^{2+} current (37.3±2.0%), an effect not seen with hypoxia and which may account for its apparent selectivity for the calcium-activated component of the K^+ current.

The mechanism by which arachidonic acid inhibition of K^+ currents occurred was investigated using pharmacological inhibitors of arachidonic acid metabolism. Pre-treatment with 1-aminobenzotriazole (3mM) a suicide substrate cytochrome P-450 inhibitor for 60-min did not alter the degree of inhibition of K^+ currents by 2μM arachidonic acid. Likewise bath application of either the cyclooxygenase inhibitor indomethacin (5μM) or the non-specific lipoxygenase inhibitor phenidone (5μM) failed to significantly affect the arachidonic acid inhibition.

These data are not supportive for a role for a cytochrome P-450 metabolite of arachidonic acid in hypoxic chemotransduction. Instead they suggest a direct, non-selective effect of arachidonic acid on the ionic currents of isolated rat type I carotid body cells.

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DEVELOPMENT OF CAUDAL HYPOTHALAMIC NEURONAL RESPONSES TO HYPOXIA IN THE RAT. Eric M. Horn and Tony G. Waldrop. Department of Molecular and Integrative Physiology, Neuroscience Program and College of Medicine, University of Illinois, Urbana, IL 61803.

Neurons in the caudal hypothalamus of adult rats have previously been shown to be stimulated by hypoxia both *in vivo* and *in vitro*. Because this region is known to be a site of cardiorespiratory regulation during hypoxia, the goal of the present study was to examine the ionic mechanisms involved in this excitation during development. Utilizing the brain slice preparation, caudal hypothalamic neurons from rats of various ages (4-39 days) were whole-cell patch clamped under voltage-clamp conditions to determine the current responses to moderate (10% O_2/85% N_2/5% CO_2) or severe (5% CO_2/95% N_2) hypoxia. Analyses of basal electrophysiological characteristics show that neonatal (4-16 days) neurons have a significantly larger resting membrane impedance, tau value, and one-half time to spike height when compared to juvenile (20-39 days) neurons in the caudal hypothalamus. Moderate hypoxia elicited a net inward current in a significantly larger percentage of caudal hypothalamic neurons from juvenile rats as compared to neonatal rats (71% vs. 44%; $p < 0.05$). In addition, a similar difference in the percentage of neurons responding to severe hypoxia was observed between juvenile and neonatal caudal hypothalamic neurons (88% vs. 62%; $p < 0.05$). While the percentages of stimulated neurons were different between the age groups, the magnitude of the inward current response to either moderate or severe hypoxia was not significantly different in caudal hypothalamic neurons between juvenile and neonatal neurons. A subset of neurons were tested independent of age for the ability to maintain the inward current response to hypoxia during synaptic blockade (11.4 mM Mg^{2+}/ 0.2 mM Ca^{2+}). Most of the neurons tested (76.9%) maintained their response, although the average current response was significantly decreased in those cells that retained the inward current response to hypoxia. Addition of the Na^+ channel blocker, tetrodotoxin (1-2 μM) in the perfusion solution following the synaptic blockade media attenuated the inward current response to hypoxia by greater than 75% ($p < 0.05$). The inward current response was unaffected by the addition of 2 mM $CoCl_2$ (general calcium channel blocker) to the bathing medium. These results indicate the excitation elicited by hypoxia in hypothalamic neurons is age dependent. In addition, the inward current response of caudal hypothalamic neurons is not dependent upon synaptic input but is due primarily to inward sodium currents (Supported by NIH 38726 and USPHS T32-GM07143).

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PROPERTIES OF IONIC CURRENTS FROM ISOLATED ADULT RAT CAROTID BODY CHEMORECEPTOR CELLS. EFFECT OF HYPOXIA

José Ramón López-López, Constancio González and María Teresa Pérez-García

The electrical properties of chemoreceptor cells from neonatal rat and adult rabbit carotid bodies (CBs) are strikingly different. These differences have been suggested to be developmental and/or species related. To distinguish between the two possibilities, the whole-cell configuration of the patch clamp technique was used to characterize the ionic currents present in isolated chemoreceptor cells from adult rat CBs. Since hypoxia-induced inhibition of O_2-sensitive K^+ currents is considered a crucial step in O_2 chemoreception, the effect of hypoxia on the adult rat chemoreceptor cell currents was also studied.

Outward currents were carried mainly by K^+, and two different components can be distinguised: A Ca^{2+}-dependent K^+ current ($IK_{(Ca)}$) sensitive to Cd^{2+} (100μM) and charybdotoxin (ChTX; 10nM) and a Ca^{2+}-insensitive, voltage-dependent K^+ current ($IK_{(V)}$). $IK_{(v)}$ showed a slow voltage-dependent activation (τ declined from 187.4 ms at -20 mV to 8.8 ms at +60 mV) and a very slow inactivation. Inactivation was described by the sum of two exponentials (τ_1= 684±150 ms, τ_2=4,956±760 ms at +30 mV) and was almost voltage-independent. The kinetic and pharmacological properties of $IK_{(V)}$ are typical of a delayed rectifier K^+ channel. Inward currents through voltage-dependent Ca^{2+} channels (I_{Ca}) were present in 19 of 27 cells (71%). TTX-sensitive Na^+ currents were also observed in about 10% of the cells. Low PO_2 produced a reduction in the outward current amplitude that averaged 22.17±1.96% (n = 27) at +20mV, being this effect absent in the presence of Cd^{2+}. Since low PO_2 did not affect I_{Ca}, we conclude that hypoxia selectively blocks $IK_{(Ca)}$. The just described properties of the currents recorded in adult rat chemoreceptor cells, including the specific inhibition of $IK_{(Ca)}$ by hypoxia, are similar to those reported in neonatal rat CB cells, impliying that the differences between rat and rabbit chemoreceptor cells are species-related.

Inhibition of $IK_{(Ca)}$ by hypoxia was further explored. Hypoxia reduced the maximal current amplitude of the Ca^{2+}-activated component of $IK_{(Ca)}$ by 70.69±4.24% and increased the apparent Ca^{2+} binding affinity by 60.81±13.61%. This latter observation implies that, at low $[Ca^{2+}]_i$, hypoxic inhibition of $IK_{(Ca)}$ is almost negligible, allowing to conclude that the inhibition of $IK_{(Ca)}$ is not likely to be the trigger of the low PO_2-chemotransduction process.

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CHARACTERISTIC FEATURES OF A NOVEL POTASSIUM ION CHANNEL FROM THE BACTERIUM *STREPTOMYCES LIVIDANS*

H. SCHREMPF[1], O. SCHMIDT[1], R. KÜMMERLEN[1], S. HINNAH[2], D. MÜLLER[2], M. BETZLER[2], T. STEINKAMP[2], and R. WAGNER[2]

[1]Angewandte Genetik der Mikroorganismen, FB Biologie/Chemie, Universität Osnabrück, 49069 Osnabrück, Germany

[2]Biophysik, FB Biologie/Chemie, Universität Osnabrück, 49069 Osnabrück, Germany

We report the identification, functional expression, purification, reconstitution, and electrophysiological characterization of an up to now unique procaryotic potassium ion channel (KcsA). It has a rectifying current voltage relation and displays subconductance states, the largest of which amounts to $\Lambda \cong 90$ pS. The *kcs*A gene has been identified in the gram-positive soil bacterium *Streptomyces lividans*. It encodes a predicted 17.6 kDa protein with two potential membrane-spanning helices linked by a central domain which shares a high degree of similarity with the H5-segment conserved among eucaryotic ion channels. Multiple alignments of deduced amino acids suggest that the novel channel has the closest kinship to the S5-, H5- and S6-regions of voltage-gated K^+-channel families, mainly to the subfamily represented by the *Shaker* protein from *Drosophila melanogaster*. Moreover, KcsA is most distantly related to eucaryotic inwardly rectifying channels with two putative predicted transmembrane segments. At present the regulation of transcription is being investigated.

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Drosophila tracheal development as a model system for angiogenesis

Pablo Wappner, Elazar Zelzer and Ben-Zion Shilo
The Weizmann Institute of Science, Rehovot (76100), Israel.

The respiratory system of insects consists of a series of interconnected tubes called tracheae that ramify to reach every single cell in the organism. Initially, during embryogenesis, tracheal development is a highly stereotyped developmental process. Nevertheless, the outgrowth of the terminal branches during the larval phases is extremely plastic and is known to depend on local oxygen concentrations at the target tissues very much alike angiogenesis in mammalian systems.

We have found that the gene *breathless*, encoding a *Drosophila* FGF receptor homologue is required not only for tracheal cell migration but also for the development of the oxygen-dependent terminal branches (1). Therefore, the paradigm is that oxygen concentrations at the target tissues may regulate the expression of a Breathless ligand in a similar way to VEGF induction in mammalian angiogenesis.

In addition, we have cloned and characterized the *trachealess (trh)* gene, a key regulator of tracheal development (2). Trh is a bHLH-PAS transcription factor that shows high homology to the mammalian Hif-1α, specially in the basic DNA-binding region. Assays to determine whether Trh or other bHLH-PAS proteins are expressed *in vivo* in response to hypoxic conditions are being developed. This will allow us in the future to use genetic tools to identify elements necessary for sensing and transduction of the hypoxic signal in *Drosophila*.

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List of Invited Speakers

Instituto Juan March (Madrid)

Workshop on

OXYGEN REGULATION OF ION CHANNELS AND GENE EXPRESSION

List of Invited Speakers

Helmut Acker	Max-Planck-Institut für Molekulare Physiologie, Rheinlanddamm 201, D-44139 Dortmund (Germany). Tel.: 49 231 1206 530 Fax : 49 231 1206 464 E-mail: helmut.acker@mpi-dortmund.mpg de
Stephen L. Archer	Veterans Affairs Medical Center and University of Minnesota, Minneapolis, MN. (USA) Tel.: 1 612 725 22 64 Fax : 1 612 727 56 68
Pierre Boistard	Laboratoire de Biologie Moléculaire des Relations Plantes-Microorganismes CNRS-INRA, BP 27, 31326 Castanet Tolosan Cedex (France). Te.l: 33 5 61 28 50 28 Fax : 33 5 61 28 50 61 E-mail: boistard@toulouse.inra.fr
Jaime Caro	Thomas Jefferson University, Jefferson Medical College, Department of Medicine, Cardeza Foundation for Hematologic Research, 1015 Walnut Street, Philadelphia, PA. 19107-5099 (USA). Tel.: 1 215 955 77 75 Fax : 1 215 923 38 36
Maria F. Czyzyk-Krzeska	Department of Molecular and Cellular Physiology, University of Cincinnati, PO Box 670576, Cincinnati, OH. 45267-0576 (USA). Tel.: 1 513 558 56 36 Fax : 1 513 558 57 38
Laura L. Dugan	Center for the Study of the Nervous System Injury, Washington University in St. Louis,Department of Neurology, Box 8111, 660 S. Euclid Ave, St. Louis, MO. 63110 (USA). Tel.: 1 314 747 04 22 Fax : 1 314 362 94 62
Alfredo Franco-Obregón	Department of Cardiology, Children's Hospital Medical Center, Harvard Medical School, 1309 Enders Building, 320 Longwood Avenue, Boston, MA. 02115 (USA) Tel.: 1 617 355 86 28 Fax : 1 617 738 95 78

Instituto Juan March (Madrid)

Constancio González	Departamento de Bioquímica y Biología Molecular y Fisiología, Facultad de Medicina, Universidad de Valladolid, 47005 Valladolid (Spain). Tel.: 34 83 42 30 85 Fax : 34 83 42 35 88
Gabriel G. Haddad	Department of Pediatrics & Cellular & Molecular Physiology, Section of Respiratory Medicine, Yale University School of Medicine, 333 Cedar Street, Fitkin Bldg. RM. 506, New Haven, CT. 06520-8064 (USA). Tel.: 1 203 785 54 44 Fax : 1 203 785 63 37 E-mail: gabriel.haddad@yale.edu
José López-Barneo	Departamento de Fisiología Médica y Biofísica, Facultad de Medicina, Universidad de Sevilla, Avda. Sánchez Pizjuán 4, 41009 Sevilla (Spain). Tel.: 34 5 455 17 68 Fax : 34 5 455 17 69 E-mail: LBARNEO@OBELIX.CICA.ES
Colin A. Nurse	Department of Biology, McMaster University, 1280 Main Street West, Hamilton, ON. Canada L8S 4K1. Tel.: 1 905 525 91 40 Fax : 1 905 522 60 66 E-mail: nursec@mcmail.CIS.McMaster.CA
Chris Peers	Institute for Cardiovascular Research, University of Leeds , Leeds LS2 9JT (U.K.). Tel.: 44 113 233 48 20 Fax : 44 113 233 48 03 E-mail: PHA6CP@south-01.novell.leeds.ac.uk
Olaf Pongs	Institut für Neurale Signalverarbeitung, Zentrum für Molekulare Neurobiologie, University of Hamburg, Martinistr. 52 - Haus 42, D-20246 Hamburg (Germany). Tel.: 49 40 47 17 50 81 Fax : 49 40 47 17 51 02
Peter J. Ratcliffe	Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford OX39DU (U.K.). Tel.: 44 1865 22 23 82 Fax : 44 1865 22 25 00
Helmut Sies	Institut für Physiologische Chemie I, Heinrich-Heine-Universität Düsseldorf, Postfach 101007, D-40001-Düsseldorf (Germany). Tel.: 49 211 311 27 07 Fax : 49 211 811 30 29

Instituto Juan March (Madrid)

Nicholas B. Standen	Ion Channel Group, Department of Cell Physiology and Pharmacology, University of Leicester, P.O. Box 138, Leicester LE1 9HN (U.K.). Tel.: 44 116 252 33 02 Fax : 44 116 252 50 45 E-mail: nbs@leicester.ac.uk
E. Kenneth Weir	VA Medical Center IIIc, University of Minnesota, 1 Veterans Drive, Minneapolis, MN. 55417 (USA). Tel.: 1 612 725 20 00 ext. 3654 Fax : 1 612 727 56 88 E-mail: weirx002@maroon.tc.umn.edu
Xiao-Jian Yuan	Department of Medicine, Division of Pulmonary and Critical Care Medicine and Department of Physiology, University of Maryland School of Medicine, Baltimore, MD. 21201 (USA). Tel.: 1 410 706 81 03 Fax : 1 410 706 81 62 E-mail: xyuan@umabnet.ab.umd.edu

Instituto Juan March (Madrid)

List of Participants

Instituto Juan March (Madrid)

Workshop on

OXYGEN REGULATION OF ION CHANNELS AND GENE EXPRESSION

List of Participants

Laura Almaraz
Departamento de Bioquímica y Biología Molecular y Fisiología, Facultad de Medicina, Universidad de Valladolid, c/Ramón y Cajal s/n°, 47005 Valladolid (Spain).
Tel.: 34 83 42 30 85
Fax : 34 83 42 35 88

Julián Aragonés
Servicios de Inmunología y Biología Molecular, Hospital de la Princesa, Universidad Autónoma, c/Diego de León 62, 28006 Madrid (Spain).
Tel.: 34 1 402 33 47
Fax : 34 1 309 24 96

Matías A. Avila
Instituto de Investigaciones Biomédicas, CSIC, Arturo Duperier 4, 28029 Madrid (Spain).
Tel.: 34 1 585 46 00
Fax : 34 1 585 45 87

Keith J. Buckler
University Laboratory of Physiology, Parks Road, Oxford OX1 3PT (U.K.).
Tel.: 44 1865 27 25 00
Fax : 44 1865 27 24 69
E-mail: keith.buckler@physiol.ox.ac.uk

Elisabeth Carpenter
Institute for Cardiovascular Research, University of Leeds, Leeds LS2 9JT (U.K.).
Tel.: 44 113 233 48 20
Fax : 44 113 233 48 03
E-mail: E.Carpenter@leeds.ac.uk

Emilio Cervantes
Departamento de Biología Vegetal, Facultad de Biología, Universidad de Salamanca, 37007 Salamanca (Spain).
Tel.: 34 23 29 44 00 Ext. 1951
Fax : 34 23 29 46 82

Mª Dolores Chiara
Dpt. Fisiología Médica y Biofísica, Facultad de Medicina, Universidad de Sevilla, Avda. Sánchez Pizjuán 4, 41009 Sevilla (Spain).
Tel.: 34 5 455 17 68
Fax : 34 5 455 17 69

Laura Conforti
Department of Molecular and Cellular Physiology, College of Medicine, University of Cincinnati, 231 Bethesda Avenue, Cincinnati, OH. 45267-0576 (USA).
Tel.: 1 513 558 56 36
Fax : 1 513 558 57 38
E-mail: conforl@ucbeh.san.uc.edu

Instituto Juan March (Madrid)

Emilio Geijo	Departamento de Fisiología, Facultad de Medicina, Universidad de Alicante, Apartado 374, 03080 Alicante (Spain). Tel.: 34 96 590 34 00 Fax : 34 96 590 39 62
Christopher J. Hatton	Institute for Cardiovascular Research, University of Leeds, Leeds LS2 9JT (U.K.). Tel.: 44 113 233 48 20 Fax : 44 113 233 48 03 E-mail: RMRCJH@Leeds.ac.uk
Eric M. Horn	Department of Molecular and Integrative Physiology, Neuroscience Program and College of Medicine, University of Illinois, Urbana, IL. 61803 (USA). Tel.: 1 217 244 18 40 Fax : 1 217 333 11 33 E-mail: e-horn@uiuc.edu
L.Eric Huang	Hematology-Oncology Division, Brigham & Women's Hospital, Harvard Medical School, Boston, MA. 02115 (USA). Tel.: 1 617 278 08 83 Fax : 1 617 739 07 48 E-mail: huang@calvin.bwh.harvard.edu
Prem Kumar	Department of Physiology, Medical School, University of Birmingham, Birmingham B15 2TT (U.K.). Tel.: 44 121 414 69 07 Fax : 44 121 414 69 24 E-mail: p.kumar@bham.ac.uk
José Ramón López-López	Departamento de Bioquímica y Biología Molecular y Fisiología, Facultad de Medicina, Universidad de Valladolid, 47005 Valladolid (Spain). Tel.: 34 83 42 30 85 Fax : 34 83 42 35 88
Gracia-P. Ortega-Sáenz	Departamento de Fisiología Médica y Biofísica, Facultad de Medicina, Universidad de Sevilla, Avda. Sánchez Pizjuan 4, 41009 Sevilla (Spain). Tel.: 34 5 455 17 68 Fax : 34 5 455 17 69
Manuel O. de Landázuri	Servicio de Inmunología, Hospital de la Princesa, Universidad Autónoma, c/ Diego de León 62, 28006 Madrid (Spain). Tel.: 34 1 402 33 47 Fax : 34 1 309 24 96
Ricardo Pardal	Departamento de Fisiología Médica y Biofísica, Facultad de Medicina, Universidad de Sevilla, Avda. Sánchez Pizjuán 4, 41009 Sevilla (Spain). Tel.: 34 5 455 17 68 Fax : 34 5 455 17 69

Instituto Juan March (Madrid)

David R. Pepper	Department of Physiology, Medical School, University of Birmingham, Birmingham B15 2TT (U.K.). Tel.: 44 121 414 69 07 Fax . 44 121 414 69 19 E-mail: d.r.pepper@bham.ac.uk
Mª Teresa Pérez-García	Departamento de Bioquímica y Biología Molecular y Fisiología, Facultad de Medicina, Universidad de Valladolid, 47005 Valladolid (Spain). Tel.: 34 83 42 30 85 Fax : 34 83 42 35 88
Ricardo Rigual	Departamento de Bioquímica y Biología Molecular y Fisiología, Facultad de Medicina, Universidad de Valladolid, 47005 Valladolid (Spain). Tel.: 34 83 42 30 85 Fax : 34 83 42 35 88
Hildgund Schrempf	Angewandte Genetik der Mikroorganismen, FB Biologie/Chemie, Universität Osnabrück, Postfach 44 69, 49069 Osnabrück (Germany). Fax : 49 541 969 28 04
Barry L. Taylor	Department of Microbiology and Molecular Genetics and Center for Molecular Biology and Gene Therapy, Loma Linda University, Loma Linda, CA. 92350 (USA). Tel.: 1 909 824 44 80 Fax : 1 909 824 40 35
Pablo Wappner	The Weizmann Institute of Science, Department of Molecular Genetics and Virology, Rehovot 76100 (Israel). Tel.: 972 8 93 42 582 Fax : 972 8 93 44 108 E-mail: lvpablo@wiccmail.weizmann.ac.il

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