



OXYGEN SENSING IN THE SHEEP ADRENAL
MEDULLA

Damien John Keating

B. App. Sc. (Hons.)

Department of Physiology

University of Adelaide

December 2002

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, the thesis contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Damien Keating,

December 2002.

ACKNOWLEDGEMENTS

There are a great number of people to whom I owe a debt of gratitude for the aid they have given me during the completion of this thesis. I will attempt here to acknowledge those people, although I fear the little space I have provided for this task will do their efforts little justice.

The guidance and knowledge which my two supervisors Michael Roberts and Grigori Rychkov have provided me over the last four years has been immeasurable and I thank you both for it. I would also like to thank my fellow doctoral students in the laboratory; Ed Aromataris, Brett Bennetts and Michael Duffield, for providing such good humor, support and advice which have all helped to make our lab such an enjoyable place to work.

Special thanks must go to my Mum and Dad who have always supported me throughout my studies and in all facets of life, and to the rest of my ever increasing family who are a constant source of strength and amusement and are the reason I always look forward to coming home. I would also like to acknowledge my new extended family in Adelaide, especially Cathy, Kim and Ben who have welcomed me from the start and provided me with a home away from home.

Finally, I wish to thank my beautiful wife Sara without whose love and support none of this would have been possible. The last few years have been trying at times on a professional basis but they have been the happiest of my life since I met you. Thankyou for all the help you have given me in so many ways, I hope I can repay you somehow in the years to come.

THESIS SUMMARY

In the fetus, prior to the development of adrenal innervation, hypoxia acts directly on the adrenal medulla to stimulate catecholamine secretion which triggers a set of physiological responses that are imperative for intrauterine survival. This direct response to hypoxia is suppressed upon development of the splanchnic innervation, but reappears if the gland is denervated. In the glomus cells of the carotid body, hypoxia evokes the release of dopamine by closing KO_2 channels, leading to membrane depolarisation, Ca^{2+} entry through voltage-gated Ca^{2+} channels and subsequent catecholamine secretion. KO_2 channels also exist in the adrenal medulla, but it is unknown whether these channels play a role in initiating the response by depolarising the cell or what intracellular mechanisms enable O_2 levels to control KO_2 channel function. The means by which adrenal gland innervation is able to suppress the direct hypoxic response also remains unclear, though it is likely due to the actions of a substance released from the nerve terminals, as the direct hypoxic response returns rapidly upon denervation of the gland.

The aims of this study therefore, were: 1) to identify the channel(s) which cause the responses to hypoxia observed in the adrenal medulla. 2) investigate the different types of Ca^{2+} channels which are present, their contribution to Ca^{2+} entry and whether any particular Ca^{2+} channels modulate K^+ channels in these cells. 3) find the intracellular pathways which transmit the decrease in extracellular O_2 levels to the membrane bound ion channels. 4) find whether the actions of opioid peptides, which are released from nerve terminals innervating the adrenal medulla, account for the suppression of the direct response upon innervation by altering either K^+ or Ca^{2+} channel function.

The whole-cell patch clamp method was utilised to measure Ca^{2+} currents, characterise the KO_2 channel(s) and identify their contribution in initiating membrane

depolarisation, to study the modulation of K^+ and Ca^{2+} currents by opioid agonists, and look at the effect of reactive oxygen species (ROS) levels on K^+ current. Fluorescence of Fluo-3 AM loaded cells was used to measure changes in intracellular Ca^{2+} levels caused by K^+ channel blockers, solutions containing high external $[K^+]_o$, or during hypoxia in the presence or absence of opioid receptor agonists.

In fetal adrenal chromaffin cells, SK and BK channels were both identified as O_2 -sensitive. During episodes of hypoxia, closure of SK channels lead to depolarisation of the cell while closure of BK channels potentiated the entry of Ca^{2+} initiated by SK channel closure. While these cells were found to contain L-, N-, P/Q- and T-type Ca^{2+} currents, there was not a specific association of Ca^{2+} influx through any one of these channels and activation of $K_{(Ca)}$ current. Disruption of mitochondrial function reduced the response of chromaffin cells to hypoxia, most likely because of a reduction in ROS production, indicating that the mitochondria act as an O_2 -sensor in these cells. The stimulation of μ - and κ -type opioid receptors decreased the hypoxia-evoked $[Ca^{2+}]_i$ increase in single cells and abolished hypoxia-induced catecholamine secretion from the whole perfused adrenal gland. It appears that alterations in the resting membrane potential and reduced activation of voltage-dependent Ca^{2+} channels accounted for the actions of these opioid agonists as both μ - and κ -type receptor activation similarly decreased Ca^{2+} current and μ -type activation increased K^+ conductance to such a degree as to offset the decrease in K^+ conductance during hypoxia. The application of apamin blocked this effect, revealing that μ -type opioid receptor activation increases SK channel conductance. As SK channels have been found to close during hypoxia and initiate membrane depolarisation, the increased opening of these channels by μ -type opioid receptor stimulation provides a logical explanation for the suppression of the direct hypoxic response upon innervation of the adrenal medulla.

THESIS SUMMARY	1
COMMONLY USED ABBREVIATIONS	8
1. HYPOXIA-EVOKED SECRETION OF CATECHOLAMINES IN THE FETAL AND POSTNATAL ADRENAL MEDULLA	13
1.1 The adrenal medulla.....	15
1.1.1 Cell types within the adrenal medulla	16
1.1.2 Innervation of the adrenal medulla	17
1.1.3 Ion channels within the adrenal medulla.....	18
1.1.3.1 Ca ²⁺ channels	19
1.1.3.1.1 T-type	20
1.1.3.1.2 L-type	21
1.1.3.1.3 N-type.....	22
1.1.3.1.4 P/Q-type	23
1.1.3.1.5 R-type.....	24
1.1.3.2 Na ⁺ channels.....	25
1.1.3.3 K ⁺ channels	26
1.2 Location and function of O₂-sensitive ion channels.....	30
1.2.1 Adrenal medulla.....	31
1.2.1.1 O ₂ sensing K ⁺ channels in adrenal chromaffin cells.....	31
1.2.1.2 Hypoxia-induced catecholamine secretion <i>in utero</i> and in the neonate.....	34
1.2.2 Carotid body	36
1.2.3 Neuroepithelial bodies	40
1.2.4 Pulmonary smooth muscle	43
1.2.5 PC-12 cells.....	45
1.2.6 O ₂ -sensitive Ca ²⁺ channels	46
1.2.7 O ₂ -sensitive Na ⁺ channels	47

1.3 Intracellular mechanisms of oxygen sensing	48
1.3.1 Membrane delimited oxygen sensing.....	50
1.3.2 NADPH oxidase	52
1.3.3 Mitochondria.....	55
1.3.4 Significance of NO and CO in oxygen chemoreception	59
1.3.4.1 Nitric oxide	59
1.3.4.2 Carbon monoxide.....	60
1.4 Regulation of catecholamine secretion.....	62
1.4.1 Cholinergic stimulation-induced secretion.....	63
1.4.1.1 Secretion associated with nicotinic stimulation.....	64
1.4.1.2 Secretion associated with muscarinic stimulation	65
1.4.2 Effects of non-cholinergic stimulation on catecholamine secretion.....	67
1.4.2.1 Opioid peptides	68
1.4.2.2 VIP and PACAP	69
1.4.2.3 Substance P	70
1.4.2.4 Angiotensin II	71
1.4.2.5 Histamine	72

2. OXYGEN SENSITIVE ION CHANNELS IN FETAL AND ADULT ADRENAL CHROMAFFIN CELLS..... 74

2.1 Introduction	75
2.2 Materials and Methods	77
2.2.1 Isolation of adrenal chromaffin cells.....	77
2.2.1.1 Fetal cells	77
2.2.1.2 Adult cells	77
2.2.2 Electrophysiology	78
2.2.3 Calcium Imaging.....	79
2.2.4 Fluorescence Immunohistochemistry.....	80

2.2.4.1 Tissue preparation	80
2.2.4.2 Immunohistochemistry	80
2.2.4.3 Cell imaging techniques	81
2.2.5 Drugs	82
2.2.6 Statistics	82
2.3 Results.....	83
2.3.1 Oxygen-sensitive K ⁺ currents in fetal adrenal chromaffin cells	83
2.3.2 Effect of hypoxia on voltage-dependent Ca ²⁺ current	83
2.3.3 Contribution of different types of K ⁺ channels to the oxygen-sensitive current.....	84
2.3.4 Measurement of reversal potential	90
2.3.5 Intracellular Ca ²⁺ measurements during hypoxia	91
2.4 Discussion	96

3. INVOLVEMENT OF MITOCHONDRIA AND NADPH OXIDASE IN THE OXYGEN SENSING MECHANISM OF OVINE ADRENAL CHROMAFFIN CELLS..... 102

3.1 Introduction	103
3.2 Methods	105
3.2.1 Isolation of adrenal chromaffin cells.....	105
3.2.2 Electrophysiology	105
3.2.3 Drugs	105
3.2.4 Statistics	105
3.3 Results.....	106
3.3.1 K ⁺ conductance changes during increased ROS exposure	106
3.3.2 Involvement of NADPH oxidase in oxygen sensing	108
3.3.3 Role of mitochondria as the oxygen sensor.....	108

3.4 Discussion	111
4. μ- AND κ-TYPE OPIOID RECEPTOR STIMULATION SUPPRESSES THE DIRECT HYPOXIC RESPONSE IN OVINE ADRENAL CHROMAFFIN CELLS.....	116
4.1 Introduction	117
4.2 Methods	119
4.2.1 Isolation of adrenal chromaffin cells.....	119
4.2.2 Electrophysiology	119
4.2.3 Calcium Imaging	120
4.2.4 Drugs	120
4.2.5 Statistics	120
4.3 Results.....	121
4.3.1 Opioid agonists and hypoxia-evoked changes in Ca^{2+} entry into AMCCs.....	121
4.3.2 Opioid agonists and K^+ currents	124
4.3.3 Effect of μ opioid agonist on the hypoxia-evoked reduction of K^+ current.....	129
4.4 Discussion	132
5. THE CONTRIBUTION OF VGCCs TO Ca^{2+} ENTRY AND ACTIVATION OF Ca^{2+}-DEPENDENT K^+ CHANNELS IN FETAL AND ADULT CHROMAFFIN CELLS.....	137
5.1 Introduction	138
5.2 Methods	140
5.2.1 Isolation of adrenal chromaffin cells.....	140
5.2.2 Electrophysiology	140

5.2.3 Calcium Imaging.....	141
5.2.4 Drugs	142
5.2.5 Statistics.....	142
5.3 Results.....	143
5.3.1 Ontogenic differences in the contribution of Ca ²⁺ channels to Ca ²⁺ influx	143
5.3.2 Ontogeny of Ca ²⁺ channel contribution to total Ca ²⁺ current	144
5.3.3 Contribution of Ca ²⁺ channel subtypes to K _(Ca) current activation	150
5.4 Discussion	153
6. GENERAL DISCUSSION	159
APPENDIX 1 : μ- AND κ-TYPE OPIOID RECEPTOR AGONISTS SUPPRESS HYPOXIA-INDUCED CATECHOLAMINE SECRETION FROM WHOLE PERFUSED FETAL ADRENAL GLAND	165
Methods.....	166
Animals.....	166
Adrenal gland perfusion.....	167
Catecholamine measurement using online analysis.....	167
Statistics.....	168
Results - Perfused fetal adrenal gland	168
Opioid agonists and hypoxia-evoked catecholamine secretion.....	168
APPENDIX 2: PUBLICATIONS ARISING FROM THIS THESIS	172
REFERENCES.....	181

COMMONLY USED ABBREVIATIONS

A B

4-AP	4-aminopyridine
A	Adrenergic
ACh	Acetylcholine
ACTH	Adrenocorticotrophic hormone
AMCC	Adrenal medullary chromaffin cells
Ang II	Angiotensin II
ANOVA	Analysis of variance
AP	Action potential
ATP	Adenosine triphosphate
BK	Large conductance calcium dependent potassium channel
BK _i	Non-inactivating BK channel
BK _s	Fast-inactivating BK channel

C D

[Ca ²⁺] _i	Intracellular calcium concentration
cAMP	Cyclic adenosine 3', 5'-monophosphate
CO	Carbon monoxide
DALDA	[D-Arg ² , Lys ⁴]-dermorphin-(1-4)-amide H-Tyr-DArg-Phe-Lys-NH ₂
DHEA	Dehydroepiandrosterone

DHP	Dihydropyridine
DMEM	Dulbecco's modified Eagles medium
DNA	Deoxyribose nucleic acid
DPDPE	[D-Pen ^{2,5}]-enkephalin
DPI	Diphenylene iodonium
DTT	Dithiotreitol

E F G

EGTA	Ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid
Enk	Enkephalin
E _{rev}	Reversal potential
ETC	Electron transport chain
FITC	Fluorescein isothiocyanate
GSH	Reduced glutathione
GSSH	Oxidised glutathione

H

HEPES	N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HPV	Hypoxic pulmonary vasoconstriction
HO	Heme oxidase
HO-1	Heme oxidase-1
HO-2	Heme oxidase-2
HVA	High voltage activating

Hz Hertz

IKL

IC₅₀ Half the maximum inhibiting concentration

IP₃ Inositol trisphosphate

IK Intermediate conductance potassium channel

I_{KN} Non-inactivating potassium current

I-V Current-voltage

[K⁺] Potassium concentration

K_{ATP} ATP sensitive potassium channel

K_(Ca) calcium-dependent potassium channel

KO₂ Oxygen-sensitive potassium channel

K_m Substrate concentration for ½ the maximum rate of the reaction

Kv Voltage-gated potassium channel

LVA Low voltage activating

MN

MΩ Megaohm

μM Micromolar

mAChR Muscarinic acetylcholine receptor

mM Millimolar

mmHg Millimeters of mercury

mRNA Messenger ribonucleic acid

ms	Millisecond
mV	Millivolt
NA	Noradrenergic
nAChR	Nicotinic acetylcholine receptor
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NDS	Normal donkey serum
NEB	Neuroepithelial body
nm	Nanometer
NPPB	5-Nitro-2-(3-phenylpropylamino) benzoic acid
NO	Nitric oxide
NOS	Nitric oxide synthase
nS	Nanosiemens

OPOR

OCT	Optimal cutting temperature compound
pA	Picoamperes
PACAP	Pituitary adenylate cyclase activating peptide
PASMC	Pulmonary artery smooth muscle cell
PBS	Phosphate buffered saline
PCMBS	P-chloromercuribenzenesulphonic acid
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C

PNMT	Phenylethanolamine N-methyl transferase
PO ₂	Partial pressure of oxygen
pS	Picosiemens
PVR	PACAP/VIP receptors
ROS	Reactive oxygen species

STUV

SEM	Standard error of mean
SGC	Small granule-containing cells
SK	Small conductance calcium-dependent potassium channel
SOD	Superoxide dismutase
SP	Substance P
STREX	Stress axis-regulated exon
TEA	Tetraethylammonium
TH	Tyrosine hydroxylase
TRITC	Tetramethylrhodamine isothiocyanate
TTX	Tetrodotoxin
V _{max}	Maximum velocity of an enzyme catalysed reaction
VGCC	Voltage-gated calcium channel
VIP	Vasoactive intestinal polypeptide