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 K_{O_2} channels, leading to membrane depolarisation, Ca^{2+} entry through voltage-gated Ca^{2+} channels and subsequent catecholamine secretion. K_{O_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 K_{O_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 K_{O_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 $[K^+]$, 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.

COMMONLY USED ABBREVIATIONS

A B

4-AP	4-aminopyridine
A	Adrenergic
ACh	Acetylcholine
ACTH	Aderenocorticotrophic 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

I K L

IC_{50}	Half the maximum inhibiting concentration
IP_3	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
KO_2	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

M N

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

O P O R

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-chloromercuribenzene sulphonic 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

S T U V

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

1. HYPOXIA-EVOKED SECRETION OF CATECHOLAMINES IN THE FETAL AND POSTNATAL ADRENAL MEDULLA

The adrenal gland is the major source of the circulating catecholamines released during periods of stress in all mammalian species. These catecholamines are crucial in triggering homeostatic changes throughout the body; alterations which usually compensate for the initial physiological disturbance. One such disturbance is a reduction in arterial blood oxygen levels, known as hypoxaemia. An adequate response mechanism to hypoxaemia is crucial for survival both in fetal and postnatal stages of life.

The means by which many postnatal mammals respond to hypoxia differs from that of the fetus prior to the development of adrenal gland innervation. In the mature animal, the carotid body is the major O_2 sensing tissue, responding to episodes of hypoxaemia by increasing carotid sinus nerve activity to the medulla oblongata. This, in turn, increases respiratory and cardiovascular output, as well as increasing catecholamine secretion from the adrenal medulla. In the fetus, before functional innervation of the adrenal gland has been established, catecholamines cannot be released during hypoxia by this neural mechanism. At this developmental stage, however, the fetus is still able to respond to episodes of hypoxaemia, as chromaffin cells of the adrenal medulla respond directly to hypoxia to trigger catecholamine release.

While the mature hypoxic response has been well studied, far less is known about how catecholamine release is activated in response to hypoxia *in utero*. It is known that once splanchnic innervation of the adrenal gland has developed, the direct response of the adrenal medulla to hypoxia is suppressed, and hypoxia-evoked catecholamine secretion occurs as a result of the neurogenic chemoreceptor reflex originating in the carotid body (Seidler and Slotkin, 1986b; Cheung, 1990). Adrenal denervation restores the direct response of the adrenal medullary chromaffin cells (AMCCs) to hypoxia (Cheung, 1990), indicating that a factor released from the nerve terminals may be responsible for the suppression of the non-neurogenic response. The action of acetylcholine on nicotinic

receptors is not responsible for the suppression of the non-neurogenic hypoxic response of the adrenal medulla as pre-treatment with the nicotinic antagonist hexamethonium does not restore the non-neurogenic response (Cheung, 1990). Other biologically active agents released from the splanchnic nerves are therefore likely to be responsible for this suppression of the direct hypoxic response.

This review will focus on the cellular mechanisms which cause non-neurogenic O_2 sensing in AMCCs by examining the current literature on these and other O_2 sensing cells. The possible causes behind the suppression of the direct hypoxic response in the adrenal medulla will also be highlighted.

1.1 The adrenal medulla

The adrenal gland contains two major parts; the outer layer of cells known collectively as the adrenal cortex and a central group of cells named the adrenal medulla. The adrenal cortex contains three distinct layers of cells known as the zona reticularis, zona fasciculata and zona glomerulosa. The zona reticularis is the inner most layer of cells and synthesises and secretes androgenic hormones (McNicol, 1992). The middle section of the adrenal cortex is the zona fasciculata, which secretes and manufactures glucocorticoids (McNicol, 1992). The zona glomerulosa is the outermost layer of the adrenal cortex and produces and releases mineralocorticoids (McNicol, 1992). While both the adrenal medulla and cortex are important components involved in homeostatic maintenance, the hypoxic response of the adrenal medulla is the focus of this review and the innervation of this tissue, the cell types it contains, and the type and function of ion channels within the adrenal medulla will be discussed in detail below.

1.1.1 Cell types within the adrenal medulla

The adrenal medulla consists of several types of catecholamine secreting cell types; small intensely fluorescent cells (also know as small granule chromaffin (SGC) cells), adrenergic chromaffin (A) cells and noradrenergic (NA) chromaffin cells, as well as non-catecholamine secreting intrinsic neurones (which are discussed in Section 1.1.2) (Langley and Grant, 1999). SGC cells in the adrenal medulla are small, at around 15 μm in diameter, rich in free polysomes and ribosomes, and contain secretory granules ranging from 100 nm to 200 nm in diameter (Kajihara *et al.*, 1978; Ho *et al.*, 1994). SGC cells have been postulated as precursors of chromaffin cells (Coupland, 1989) and are thought to release noradrenaline (Langley and Grant, 1999). Their role remains unclear but SGC cells are known to respond to insulin-induced hypoglycaemia in a similar fashion to NA cells (Coupland *et al.*, 1984). These SGC cells represent approximately 5% of the mouse AMCC population but they are more scarce in the majority of species including cat, dog, rabbit, rat and guinea-pig (Coupland, 1989).

AMCCs are by far the more abundant cell type compared to SGC cells and intrinsic neurones and are the main cell type responsible for the supply of the circulating catecholamines, adrenaline and noradrenaline, in vertebrates. The relative proportions of A and NA cells varies greatly among species, but in most cases A cells predominate. In the bovine adrenal gland, A and NA-chromaffin cells are present in an approximate ratio of 3:1 which corresponds to the ratio of adrenaline to noradrenaline contained in bovine adrenal glands (Fenwick *et al.*, 1978). This ratio varies during fetal development with a far greater number of noradrenaline-secreting cells present in early gestation and the number of adrenaline-secreting cells increasing in late gestation (McMillen *et al.*, 1988). A and NA cells can be distinguished by electron microscopy; typical NA cells contain vesicles with cores contracted from the vesicle membrane and A cells have vesicles which evenly fill the

cytoplasm (Fenwick *et al.*, 1978) and have a lower electron density than the NA granules (Kobayashi and Coupland, 1993). Both A and NA cells are typically of equal size, round and approximately 40-60 μm in diameter (Langley and Grant, 1999), and contain secretory granules with an average diameter of around 350 nm (Plattner *et al.*, 1997).

1.1.2 Innervation of the adrenal medulla

The use of nerve degeneration and cholinesterase techniques in several studies has identified mainly preganglionic cholinergic sympathetic fibres arising from the splanchnic nerve which project to the mammalian adrenal medulla (Robinson *et al.*, 1977; Coupland *et al.*, 1989; Parker *et al.*, 1990). Most of these preganglionic cholinergic sympathetic fibres arise from the spinal cord at levels T8-T11 (Hollinshead, 1937; Holets and Elde, 1982; Kesse *et al.*, 1988; Parker *et al.*, 1993). In many mammalian species, the pattern of adrenal gland innervation is very similar, as cholinergic fibres run from a network within the adrenal capsule directly towards the centre of the gland through the cortex with only slight branching until they reach the medulla. Once at the medulla, widespread branching of fibres occurs to form a medullary plexus that has multiple axons connecting to single AMCCs (Robinson *et al.*, 1977; Parker *et al.*, 1993). In the rat, the ratio of preganglionic sympathetic neurons to AMCCs is approximately 1:1000 (Coupland *et al.*, 1989).

Some intrinsic innervation of the adrenal gland is also present in the form of ganglion cells located under the adrenal capsule and among cortical and medullary cells (Unsicker, 1971; Watanabe *et al.*, 1990). The number of ganglion cells is species specific and these cells are thought to originate from neural crest cells that migrated into the cortical anlage during development (Parker *et al.*, 1993).

There are at least two types of intrinsic ganglion cells in the adrenal gland, classified as type I or type II depending on their size, location and transmitter phenotype. Type I ganglion cells are larger, generally grouped in the centre of the medulla, have a postganglionic, noradrenergic phenotype and innervate not only the medulla, but project to the cortex as well (Holgert *et al.*, 1996). Type II ganglion neurons are smaller, located peripherally, usually observed as single cells or small groups of cells, and appear to innervate both chromaffin cells, cortical cells and other ganglion cells within the adrenal gland (Holgert *et al.*, 1998). These type II ganglion cells have an unusual transmitter phenotype in that they produce nitric oxide synthase (NOS) and vasoactive intestinal peptide (VIP) (Holgert *et al.*, 1995). The physiological function of these intrinsic ganglion cells remains uncertain, but their actions may involve regulation of adrenal blood flow and also secretory output from the adrenal gland.

1.1.3 Ion channels within the adrenal medulla

AMCCs are electrically excitable endocrine cells which require changes in ion movement across the cell membrane in order for catecholamine secretion to occur. This is because catecholamine exocytosis is a highly Ca^{2+}-dependent process, requiring a substantial increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) which is usually acquired from the extracellular fluid. K^+ channels largely determine the electrical potential difference across the plasma membrane and also modulate the duration and frequency of action potentials (AP). Na^+ channels are responsible for the fast inward current associated with the firing of AP and the blockade of these channels reduces the AP amplitude in AMCCs (Hollins and Ikeda, 1996). Voltage-gated Ca^{2+} channels (VGCCs) open during membrane depolarisation to allow the entry of Ca^{2+} into the cytosol and the blockade of

Ca^{2+} channels does not alter the resting membrane potential of AMCCs or prevent these cells from firing AP (Hollins and Ikeda, 1996).

1.1.3.1 Ca^{2+} channels

Entry of Ca^{2+} into the cytosol is crucial in order for exocytosis of catecholamines from AMCCs to take place and the opening of VGCCs upon membrane depolarisation is the main route of this Ca^{2+} entry. VGCCs are classified according to a number of criteria including subunit composition, biophysical properties and pharmacology. Some of these factors are outlined in Table 1.

Each VGCC contains an α_1 subunit which controls voltage-dependence and forms the Ca^{2+}-selective pore (Fisher and Bourque, 2001), one of four types of β subunits which regulate channel expression and biophysical properties (Birnbaumer *et al.*, 1998) and one of at least three different $\alpha_2\delta$ subunits (Gao *et al.*, 2000). AMCCs contain a number of different VGCC types per cell, as illustrated in bovine AMCCs where α_1 subunit mRNA for L-, N-, P/Q-, R- and T-type channels along with mRNA for the auxiliary subunits β_2, β_3, β_4, and $\alpha_2\delta$ have been identified (Garcia-Palomero *et al.*, 2000). There is a high degree of species variability associated with the exact proportion of each of these channel subunits expressed in AMCCs.

Channel subtype		α_1 subunit designation		Antagonists
		New nomenclature	Old nomenclature	
High voltage activated	L-type	$\alpha_1$1.1 $\alpha_1$1.2 $\alpha_1$1.3 $\alpha_1$1.4	α_{1S} α_{1C} α_{1D} α_{1F}	dihydropyridines
	P/Q-type	$\alpha_1$2.1	α_{1A}	ω-Agatoxin IVA ω-Agatoxin TK
	N-type	$\alpha_1$2.2	α_{1B}	ω-Conotoxin GIVA
	R-type	$\alpha_1$2.3	α_{1E}	SNX-482
Low voltage activated	T-type	$\alpha_1$3.1 $\alpha_1$3.2 $\alpha_1$3.3	α_{1G} α_{1H} α_{1I}	mibefradil, amiloride

Table 1: Nomenclature of α_1 subunits and antagonists of VGCCs.

1.1.3.1.1 T-type

These channels are categorised as low voltage activating (LVA) Ca^{2+} channels due to their low threshold of activation at around -60 mV (Fisher and Bourque, 2001) in contrast to the high voltage activated (HVA) Ca^{2+} channels, which have an activation threshold at approximately -40 mV in AMCCs (Gandia *et al.*, 1993). The inactivation of T-type channels is highly voltage-dependent, becoming increasingly rapid at more positive potentials. Channels containing the $\alpha_1$3 subunit, or those blocked by compounds such as mibefradil and amiloride (Martin *et al.*, 2000), are classified as T-type Ca^{2+} channels.

Although T-type currents are transient and of small amplitude compared to HVA currents, they are able to carry a disproportionately large fraction of the total Ca^{2+} influx during brief AP waveforms (McCobb and Beam, 1991). Despite the fact that 54% of fetal

rat (Bournaud *et al.*, 2001), 23% of adult rat (Hollins and Ikeda, 1996) and 70% of bovine (Diverse-Pierluissi *et al.*, 1991) AMCCs tested contain T-type currents, there is no evidence of this channel being involved in catecholamine secretion. T-type channels are, however, involved in the slow depolarisations which underlie burst firing activity (Huguenard, 1996), as well as pacemaker activity and neuronal firing (Chemin *et al.*, 2002) in many neurons.

1.1.3.1.2 L-type

One of the family of HVA Ca^{2+} channels, L-type channels can be distinguished from other VGCCs, especially the functionally similar N-type channel, by pharmacological blockade with dihydropyridine (DHP) antagonists and current enhancement by DHP agonists (Nowycky *et al.*, 1985). L-type channels are composed of one of three α subunits, known as $\alpha_1 1.1$, $\alpha_1 1.2$ and $\alpha_1 1.3$. L-type channels undergo Ca^{2+}-dependent inactivation (Fisher and Bourque, 2001) mediated by calmodulin which is tightly associated with the carboxy terminus of the channel (Peterson *et al.*, 1999).

A variety of species including human (Gandia *et al.*, 1998), cat (Albillos *et al.*, 1994), bovine (Albillos *et al.*, 1996), rat (Gandia *et al.*, 1995), pig (Kitamura *et al.*, 1997), mouse (Hernandez-Guijo *et al.*, 1998) and sheep (Adams *et al.*, 1996) contain this channel type within AMCCs. Hence, it is not surprising to find that L-type Ca^{2+} channels are involved in modulating catecholamine secretion from the bovine (Lomax *et al.*, 1997), rat (Kim *et al.*, 1995) and sheep (Adams *et al.*, 1996) adrenal medulla. In bovine AMCCs, L-type channels are preferentially involved in Ca^{2+} entry and catecholamine secretion compared to other Ca^{2+} channels, and this involvement is greater in noradrenergic, rather than adrenergic cells (Lomax *et al.*, 1997). Voltage steps to +10 mV from a holding

potential of -70 mV produce both inward Ca^{2+} currents and catecholamine secretion, with each reduced by L-type channel blockers and enhanced by L-type channel agonists by 35-40% in rat AMCCs (Kim *et al.*, 1995). L-type Ca^{2+} channels appear closely associated with hypoxia-induced catecholamine secretion from whole perfused adrenal glands of fetal sheep, as this secretion is abolished by the specific L-type Ca^{2+} channel antagonist, nifedipine (Adams *et al.*, 1996).

1.1.3.1.3 N-type

N-type Ca^{2+} channels contain the $\alpha_1 2.2$ subunit, and are selectively blocked by the toxin ω-Conotoxin GVIA which is isolated from the venom of the marine snail *Conus geographicus*. N-type Ca^{2+} channels can vary from rapidly inactivating to non-inactivating in different cell types. The blockade of these channels partially inhibits neurotransmitter release from certain neurones (Kamiya *et al.*, 1988; Dutar *et al.*, 1989; Horne and Kemp, 1991) and they have been localised at the neuromuscular junction (Robitaille *et al.*, 1990). They are present in AMCCs of the same species as L-type channels mentioned previously (Albillos *et al.*, 1994; Gandia *et al.*, 1995; Albillos *et al.*, 1996; Kitamura *et al.*, 1997; Gandia *et al.*, 1998; Hernandez-Guijo *et al.*, 1998), and like L-type channels, the proportion of N-type channels present in these cells varies widely between species.

In cultured rat AMCCs, N-type channels contribute significantly to secretion triggered by depolarising voltage steps (Kim *et al.*, 1995) and while they also play a role in exocytotic control during sustained splanchnic nerve stimulation of the whole perfused adrenal, they are not significantly involved in secretion generated by short duration stimuli (O'Farrell *et al.*, 1997; Santana *et al.*, 1999). In cultured single bovine AMCCs, pharmacological blockade of N-type channels does not effect secretion induced by high

external [K^+] (Lomax *et al.*, 1997) but decreases secretion evoked by G protein-coupled purinoceptor stimulation (Powell *et al.*, 2000). These results illustrate the effect which cell culture and the type of stimulation can have when interpreting the level of involvement of particular sub-types of Ca^{2+} channels in catecholamine secretion.

1.1.3.1.4 P/Q-type

P-type current were named as such because they were first described in cerebellar Purkinje cells where a HVA current insensitive to block by either dihydropyridines or ω-Conotoxin GVIA almost totally constitutes the Ca^{2+} current (Llinas *et al.*, 1989). Currents produced by P-type channels are generally non-inactivating and are blocked specifically by a peptide component of the venom from funnel web spiders, *Agelenopsis aperta*, known as ω-Agatoxin IVA (IC_{50} = 2 nM) (Mintz *et al.*, 1992). A Ca^{2+} current demonstrating similar biophysical properties and slightly less sensitivity to this toxin (IC_{50} = 200 nM) was later found in cerebellar granule cells and named Q-type current (Zhang *et al.*, 1993).

Both P- and Q-type Ca^{2+} channels contain the $\alpha_1 2.1$ subunit and they are thought to differ only in the β subunit with which they are coexpressed (Fisher and Bourque, 2001). However, since the range of biophysical properties of native ω-Agatoxin-sensitive P- and Q-type Ca^{2+} channels are very similar, and subunit composition of these channels is unknown *in situ*, they are regularly denoted as P/Q-type channels.

A variety of species including human (Gandia *et al.*, 1998), cat (Albillos *et al.*, 1994), bovine (Albillos *et al.*, 1996), rat (Gandia *et al.*, 1995), pig (Kitamura *et al.*, 1997) and mouse (Hernandez-Guijo *et al.*, 1998) contain P/Q-type channels in their AMCCs. In bovine AMCCs, catecholamine secretion is controlled by both L- and Q-type channels (Lomax *et al.*, 1997) with Q-type channels located closer to secretory sites than L-type

(Lara *et al.*, 1998). L- and P/Q-type, but not N-type channels, modulate secretion in whole perfused rat adrenals during stimulation of the splanchnic nerve and by cholinergic agonists (Santana *et al.*, 1999). In 'tottering' mice, which carry a mutation of the $\alpha_1$2.1 subunit gene, the amount of catecholamine secretion evoked by high extracellular K^+ from the AMCCs of these mice is not significantly different from that of wild-type mice (Colliver *et al.*, 2001). This is in agreement with previous results from rat AMCCs which illustrated that L- and N-type Ca^{2+} channels contribute wholly to catecholamine secretion induced by depolarising voltage steps (Kim *et al.*, 1995).

1.1.3.1.5 R-type

The most recently classified Ca^{2+} channel group, R-type Ca^{2+} channels are HVA channels named from their "resistance" to block by all of the previously mentioned Ca^{2+} channel antagonists. The $\alpha_1$2.3 subunit is thought to be the α subunit for R-type channels because heterologously expressed $\alpha_1$2.3 channels have similar fast inactivation kinetics and pharmacological properties to R-type channels (Ellinor *et al.*, 1993). The mRNA for the $\alpha_1$2.3 subunit of R-type Ca^{2+} channels has been identified in bovine AMCCs (Garcia-Palomero *et al.*, 2000). A blocker of this current, SNX-482, has been recently isolated from the venom of the African tarantula, *Hysterocrates gigas* (Newcomb *et al.*, 1998).

Direct evidence for a role of R-type channels in neurotransmission has been difficult to obtain mainly due to the absence, until recently, of a selective antagonist. Usually this channel is implicated as being present in a cell due to a lack of effect of L-, N- or P/Q-type channel antagonists, as in the case of inhibitory synapses innervating rat Purkinje cells where these blockers are not able to inhibit this type of synaptic input (Doroshenko *et al.*, 1997). R-type currents, again defined by resistance to blockade by L-,

N- and P/Q-type channel antagonists, are responsible for over half the secretory output occurring due to short depolarising pulses in mouse adrenal slices (Albillos *et al.*, 2000) and approximately 10% of Ca^{2+} current and catecholamine secretion in cultured mouse AMCCs (Aldea *et al.*, 2002). It would appear from these results that either the effect of culturing AMCCs or the type of stimulation applied to adrenal slices or cultured cells are factors which can alter the level of contribution R-type channels make towards secretion and Ca^{2+} entry. As it has been observed that not all currents resistant to L-, N- or P/Q-type channel blockade are R-type, and SNX-482 is not totally specific for R-type channels (Fisher and Bourque, 2001), care must be taken in classifying R-type channels solely on a pharmacological basis.

1.1.3.2 Na^+ channels

The role of Na^+ channels in the regulation of cellular functions is a vital one, due mainly to their involvement in the production of AP. Voltage-gated Na^+ channels have been identified in all excitable cells of the mammalian body, including AMCCs (Cui and Pun, 1994; Lopez *et al.*, 1995; Hollins and Ikeda, 1996; Yanagita *et al.*, 2000). Na^+ channels in rat AMCCs are responsible for the fast inward current with rapid activation and inactivation kinetics which activates at around -45 mV, peaks at -20 mV and has an estimated peak amplitude of around 6 nA in physiological solution (Hollins and Ikeda, 1996). The opening of Na^+ channels by the agonist, veratridine, induces large oscillations in $[Ca^{2+}]_i$ and membrane potential in otherwise silent bovine AMCCs (Lopez *et al.*, 1995).

Na^+ channels in AMCCs are modulated by several factors including angiotensin II, which blocks Na^+ channels in bovine AMCCs (Cui and Pun, 1994). Protein kinase C (PKC) down-regulates the level of cell surface Na^+ channel expression without altering the

gating of Na^+ channels (Yanagita *et al.*, 2000). This is because the PKC isoform, cPKC-α, promotes Na^+ channel internalisation, and nPKC-ε decreases the Na^+ channel α-subunit mRNA level by shortening the half-life of the α-subunit mRNA (Yanagita *et al.*, 2000). While blockade of Na^+ channels decreases AP amplitude it does not alter resting membrane potential (Hollins and Ikeda, 1996).

1.1.3.3 K^+ channels

Different K^+ channels can be distinguished, like may other ion channels, on the basis of activation and inactivation kinetics, voltage dependence and sensitivity to pharmacological blockade. AMCCs fire AP due to the actions of voltage-dependent Na^+, Ca^{2+} and K^+ channels, with the frequency of AP firing being greatly enhanced by the nicotinic agonist, acetylcholine (Brandt *et al.*, 1976). The gating of K^+ channels has the greatest effect on resting membrane potential, action potential firing and the length of the afterhyperpolarisation, all of which effect the level of catecholamine secretion from AMCCs. This section will review the K^+ channels present in AMCCs and their role in controlling membrane potential, action potential firing and secretion.

Adult and fetal AMCCs, across a number of species, contain a variety of K^+ channel types, and the first investigations into the different classes of K^+ channels present in AMCCs were carried out in cultured bovine cells (Marty and Neher, 1985). This study found three distinct K^+ current types, one of which was described as a Ca^{2+}-dependent K^+ ($K_{(Ca)}$) channel with large unitary conductance and identified as that produced by large conductance, voltage-gated, Ca^{2+}-activated K^+ (BK) channels. Later investigations confirmed the presence of TEA-sensitive BK channels in rat (Neely and Lingle, 1992;

Solaro *et al.*, 1995; Prakriya *et al.*, 1996; Lovell *et al.*, 2000), dog (Nagayama *et al.*, 1998), sheep (Rychkov *et al.*, 1998) and bovine (Wada *et al.*, 1995) AMCCs.

The exact type and function of BK channels within the adrenal medulla *in vivo* may vary due to the actions of steroid hormones. mRNA expression levels of the *Slo* gene, the only gene known to encode BK channels, is not altered in the rat adrenal medulla 10 weeks after hypophysectomy (Xie and McCobb, 1998). However, the ratio of two splice variants of the *Slo* gene, one with the STREX (STRess axis-regulated EXon) exon inserted in the C-terminus and the other without this insert, named ZERO, does change, resulting in a reduced level of STREX compared to ZERO, an effect reversed upon a series of subcutaneous injections of ACTH (Xie and McCobb, 1998). When both the STREX and ZERO splice variant of BK channels are expressed in *Xenopus laevis* oocytes, currents from STREX injected oocytes activate at voltages approximately 20 mV negative to those from ZERO-injected oocytes, have a half-activation voltage of -2 mV compared to +20 mV and display faster activation and slower deactivation kinetics than ZERO currents (Xie and McCobb, 1998). Slower deactivation of BK currents has been linked to enhanced repetitive firing in AMCCs (Solaro *et al.*, 1995) as the increased afterhyperpolarisation facilitates recovery from inactivation in Na^+ and Ca^{2+} channels (Xie and McCobb, 1998). Faster BK channel activation and activation at more negative potential will further augment the afterhyperpolarisation by increasing BK channel openings during AP firing. This is illustrated by hypophysectomy causing a 50% decrease in the maximum number of spikes elicited with a 2 second depolarising current pulse in rat AMCCs (Lovell and McCobb, 2001).

The direct application of ACTH to cultured bovine AMCCs has no effect on STREX inclusion, illustrating that ACTH does not act on AMCCs directly (Lai and McCobb, 2002). As ACTH is known to stimulate glucocorticoid secretion from

steroidogenic cells in the adrenal cortex, it was postulated that the actions of corticosteroids were responsible for the indirect effect of ACTH on STREX splicing in rat AMCCs (Xie and McCobb, 1998). Contrary to this hypothesis, cortisol and the synthetic corticosteroid dexamethasone accelerate the decline in STREX levels in bovine cell cultures, an effect blocked by the specific glucocorticoid receptor antagonist, RU38468 (Lai and McCobb, 2002). Like cortisol, the release of the androgen steroid, dehydroepiandrosterone (DHEA), is also stimulated by pituitary ACTH (Cutler *et al.*, 1979; Albertson *et al.*, 1984) and the application of DHEA, testosterone or androstenedione to cultured bovine AMCCs increases STREX levels (Lai and McCobb, 2002). These results indicate that steroid hormones, including glucocorticoids and androgens, released from the adrenal cortex, regulate STREX splicing of BK channels and mediate AMCC excitability and possibly catecholamine secretion.

The presence of these BK channel splice variants is thought to underlie the differences in BK channel types seen within the same species. In rat AMCCs, two variants of BK currents are observed, one being non-inactivating with slower channel deactivation (named BK_i) and the other having fast-inactivation kinetics (named BK_s) (Solaro *et al.*, 1995). The differences in kinetics cannot be explained by differences in cytosolic $[Ca^{2+}]$ regulation, the activation time course or the dependence of activation on $[Ca^{2+}]_i$ and membrane potential (Solaro *et al.*, 1995). Both current types contribute to the action potential repolarisation but the cells expressing BK_i fire action potentials more repetitively during constant current injections (Solaro *et al.*, 1995). Similar BK currents have been found in bovine AMCCs also, however the proportion of cells displaying only BK_i is reduced from 75% in rat (Solaro *et al.*, 1995) to 5% in bovine AMCCs (Lovell *et al.*, 2000). The characteristics of BK_s and BK_i currents closely matches those described for ZERO and STREX BK currents, respectively.

Hypoxia reduces the amplitude of a BK-type K^+ current in fetal sheep AMCCs (Rychkov *et al.*, 1998) but it is unknown whether these channels control the resting membrane potential as blockade of BK channels in the whole perfused adrenal of dogs does not initiate secretion (Nagayama *et al.*, 1997). Blockade of these channels does potentiate secretion evoked by nicotinic stimulation in bovine AMCCs (Wada *et al.*, 1995) and BK channels are opened by muscarine-induced elevations in $[Ca^{2+}]_i$ in rat AMCCs (Prakriya *et al.*, 1996).

Apamin-sensitive small conductance Ca^{2+}-activated K^+ (SK) channels are also found in rat (Neely and Lingle, 1992; Solaro *et al.*, 1995), dog (Nagayama *et al.*, 1997) and bovine (Artalejo *et al.*, 1993; Wada *et al.*, 1995) AMCCs. Unlike BK channels, SK channels are not voltage-dependent, but rely solely on intracellular Ca^{2+} to increase their open probability (Neely and Lingle, 1992). SK channels are responsible for the slow afterhyperpolarisation current which occurs after the firing of an action potential (Vergara *et al.*, 1998), as this is when $[Ca^{2+}]_i$ is elevated. The current produced by these channels accounts for most of the membrane current at potentials negative to -40 mV (Neely and Lingle, 1992) and specific blockade of these channels with apamin causes a membrane depolarisation of around 20 mV (Lee *et al.*, 2000). Interestingly, SK channel blockade causes repetitive AP firing in rat AMCCs which contain BK_i channels, but not in those with BK_s channels (Solaro *et al.*, 1995).

Blockade of SK channels augments catecholamine secretion in bovine AMCCs during stimulation with histamine (Lara *et al.*, 1995; Kitamura *et al.*, 1996), the nicotinic agonists DMPP (Lara *et al.*, 1995), and carbachol (Wada *et al.*, 1995). In the whole perfused adrenal gland, SK channel blockade enhances catecholamine secretion caused by cholinergic, muscarinic and electrical stimulation in the cat (Uceda *et al.*, 1992; Uceda *et*

al., 1994; Montiel *et al.*, 1995) and by cholinergic, but not electrical, stimulation in the dog (Nagayama *et al.*, 1997).

In bovine AMCCs a G protein-activated K^+ current which is not Ca^{2+}-dependent and is only slightly sensitive to apamin and charybdotoxin has also been identified. This K^+ current is, however, activated by fluoride in a G protein-dependent manner and the application of this compound results in a reduced level of nicotine-induced catecholamine secretion (Cannon *et al.*, 1994). A voltage-dependent, 4-AP-sensitive K^+ current has also been reported to be present in a subset of fetal sheep AMCCs (Rychkov *et al.*, 1998). It was not made clear in this study, however, whether the cells containing these channels were AMCCs or cortical cells contaminating the medullary primary cell culture, as the inactivation kinetics and sensitivity to 4-AP of this K^+ current are characteristic of those observed in cortical cells of the zona fasciculata (Barbara and Takeda, 1995).

1.2 Location and function of O_2-sensitive ion channels

The existence of oxygen-sensitive potassium (K_{O_2}) channels and their physiological role was first uncovered in carotid body glomus cells through the pioneering work of the group headed by Jose Lopez-Barneo (Lopez-Barneo *et al.*, 1988). This initial paper utilised electrophysiological techniques to illustrate the presence of K_{O_2} channels and since then this group has demonstrated that hypoxia depolarises the glomus cell membrane, opening VGCCs and causing Ca^{2+} entry and neurotransmitter release (Lopez-Lopez *et al.*, 1989; Ganfornina and Lopez-Barneo, 1991; Ganfornina and Lopez-Barneo, 1992; Lopez-Barneo, 1993; Urena *et al.*, 1994; Pardal *et al.*, 2000). While the model of K^+ channel closure, membrane depolarisation and Ca^{2+} entry is now known to be the general response mechanism to hypoxia in all O_2 sensing cells, the major differences between these cells are

the diversity of K^+ channels responsible for transducing changes in plasma O_2 levels and the apparent identity of the O_2 sensor within these cells. The latter will be discussed in the following section, while the identity of various K_{O_2} channels in different O_2 sensing cell types; namely the adrenal medulla, carotid body, pulmonary smooth muscle, neuroepithelial body and PC-12 cells, will be addressed below. As well as this, different types of O_2-sensitive Ca^{2+} and Na^+ channels in a variety of cell types will be reviewed.

1.2.1 Adrenal medulla

1.2.1.1 O_2 sensing K^+ channels in adrenal chromaffin cells

A wide variety of K_{O_2} channels have been identified in a number of O_2-sensitive tissues in mammals. Briefly, BK (Peers, 1990; Wyatt *et al.*, 1995; Hatton *et al.*, 1997) and TASK-1 channels (Buckler, 1999; Buckler *et al.*, 2000) are O_2-sensitive in rat glomus cells. In PC-12 cells, a slowly inactivating K^+ current is inhibited by hypoxia (Zhu *et al.*, 1996; Conforti and Millhorn, 1997) and the channel producing this current has been identified as the Kv1.2 channel (Conforti and Millhorn, 1997). In neuroepithelial bodies, K^+ currents produced by Kv3.3 channels are O_2-sensitive (Youngson *et al.*, 1993; Wang *et al.*, 1996; Fu *et al.*, 1999), while the neuroepithelial body-derived cell line, H-146, contain O_2-sensitive TASK-3 channels (Hartness *et al.*, 2001). Pulmonary artery smooth muscle cells are also O_2-sensitive due to the actions of TASK-1 channels (Gurney *et al.*, 2002) and possibly a K^+ channel from the Kv channel family (Hulme *et al.*, 1999; Osipenko *et al.*, 2000).

Less is known of the identity of the K_{O_2} channel in AMCCs compared to the K_{O_2} channels of the other oxygen sensing tissues mentioned above. This is because hypoxic

suppression of K^+ current was only recently discovered in neonatal rat (Thompson *et al.*, 1997), fetal sheep (Rychkov *et al.*, 1998) and adult rat (Lee *et al.*, 2000) AMCCs.

It had been thought that a K_{O_2} channel was responsible for the hypoxia-induced membrane depolarisation (Inoue *et al.*, 1998) and Ca^{2+} entry which had been observed in AMCCs (Mochizuki-Oda, 1997; Mojet *et al.*, 1997) and led to catecholamine secretion in both single AMCCs (Mochizuki-Oda, 1997; Mojet *et al.*, 1997; Inoue *et al.*, 1998) and the whole perfused adrenal gland (Adams *et al.*, 1996). The first investigation into identifying the K^+ channel responsible for the hypoxic response in sheep AMCCs found two different O_2-sensitive channel types. These were a fast inactivating, 4-AP-sensitive K^+ current with voltage-dependent inactivation and a Ca^{2+}-dependent, TEA-sensitive K^+ current with the kinetics of a delayed rectifier (Rychkov *et al.*, 1998). These two current types are not present in the same population of cells with approximately 40% of cells in culture containing the fast-inactivating current. Both cell types have the same resting membrane potential and level of current reduction by hypoxia, but after 24 hours incubation at 37°C, cells displaying the fast inactivating K^+ current were no longer present (Rychkov *et al.*, 1998). Whether this is due to alterations in K^+ current expression in culture, or if these currents are contained in a separate subgroup of cells, for example noradrenaline and adrenaline-containing AMCCs, and one of these subgroups does not survive this incubation time, remains unknown.

The majority of studies on O_2 sensitivity of AMCCs have been carried out on the rat. While an increase in either $[Ca^{2+}]_i$ or catecholamine secretion is reportedly seen only once P_{O_2} falls below 5 mmHg (Mojet *et al.*, 1997), more moderate hypoxic levels up to 40 mmHg are capable of causing membrane depolarisation and reductions in K^+ current amplitude in rat neonate (1-2 days old) AMCCs (Thompson *et al.*, 1997). Anoxia acts on

these cells to reduce a $K_{(Ca)}$ current and a Ca^{2+}-independent, delayed rectifier K^+ current while at the same time activating a K_{ATP} current (Thompson and Nurse, 1998).

The O_2 sensing mechanisms found in the rat neonate do not exist in juvenile rats (2-4 weeks old) (Mojet *et al.*, 1997; Thompson *et al.*, 1997) implying a developmentally regulated O_2 sensing mechanism in these cells. This is in agreement with *in vivo* results in which hypoxia causes depletion of adrenal catecholamines in 1-day-old neonate rats via direct stimulation of the adrenal medulla by reduced P_{O_2} and in 8-day-old rats by means which require nervous input (Seidler and Slotkin, 1985). This ontogenetic replacement of the non-neurogenic hypoxic response with the neurogenic mechanism is directly related to the onset of splanchnic nerve function in the rat.

Two other investigations have found, however, that adult (7-10 weeks old) rat AMCCs are O_2-sensitive. In about 50% of these cells hypoxia decreases outward K^+ current, depolarises the membrane potential by approximately 10 mV (Lee *et al.*, 2000), increases $[Ca^{2+}]_i$ and enhances catecholamine secretion (Mochizuki-Oda, 1997). The K^+ channel responsible for these hypoxic responses was not identified in one study (Mochizuki-Oda, 1997) and identified as an SK channel in the other (Lee *et al.*, 2000).

It appears from these results that both neonate and adult, but not juvenile, rat AMCCs are capable of sensing and responding to decreased levels of P_{O_2}. It has been proposed that the onset of innervation is responsible for this difference in O_2 sensing capabilities between neonates and juveniles, but this does not explain how adult rat AMCCs are capable of responding to hypoxia. Neither differences in the rat strains used, the time cells spend in culture, nor the enzymes used to disperse the cells from the native tissue appear to be the cause of this disparity. Juvenile AMCCs were all incubated with their respective enzymes for one hour, however, which is longer than the incubation time of the adult cells (25-45 min) and indicates that enzyme exposure may have a detrimental,

time-dependent effect on O_2 sensitivity in these cells. This is strengthened by recent evidence of similar hypoxia-induced $[Ca^{2+}]_i$ increases in undigested adrenal medulla slices from rats at ages before (1-2 days and 3-6 days) and after (8-11 days, 3-5 weeks and 6-11 weeks) innervation of the adrenal gland has been established (Takeuchi *et al.*, 2001). Thus, different length of enzyme exposure, rather than ontogenic differences, may explain the variation seen in the sensitivity to hypoxia of neonate, juvenile and adult dispersed rat AMCCs.

1.2.1.2 Hypoxia-induced catecholamine secretion *in utero* and in the neonate

Catecholamines in the fetus are released in response to stressors such as hypoxia and hypoglycaemia, and are required for the development of the lung, thermogenesis and the triggering of physiological changes which occur during parturition and labour. It is well established that catecholamines in physiological concentrations are unable to cross the placenta and, therefore, catecholamines in the fetal blood must be produced by the fetus itself. The most common and serious stress to the fetus is inadequate delivery of oxygen. Fetal hypoxia can be caused in a number of ways including maternal hypoxia, umbilical cord occlusion, placental haemorrhage and decreased uterine arterial blood flow. The ability of fetal chromaffin tissue to respond to episodes of hypoxia was first demonstrated in the fetal lamb where, from as early as 80 days gestation, hypoxia can directly stimulate the adrenal medulla (Comline and Silver, 1961). Further work confirmed this rise in catecholamine levels during hypoxia, showing a forty fold increase in fetal plasma noradrenaline and adrenaline in response to hypoxia in fetal lambs (Jones and Robinson, 1975).

Various experimental methods of inducing fetal hypoxia including maternal hypoxia (Reuss and Rudolph, 1980), fetal haemorrhage (20% decrease in fetal blood volume) (Itskovitz *et al.*, 1982) and umbilical cord compression (Itskovitz *et al.*, 1987) all produce similar physiological effects. These changes include decreased heart rate, a rise in fetal arterial pressure and dramatic increases in blood flow to the heart, brain and adrenal glands with a concomitant reduction in blood flow to the kidneys, gut, liver, lung and carcass (Teitel and Rudolph, 1985). This redistribution of cardiac output during hypoxia is caused by peripheral vasoconstriction, which is also observed following fetal infusion of noradrenaline (Dawes *et al.*, 1968). This peripheral vasoconstriction is due to α–adrenergic receptor stimulation by catecholamines, as pharmacological blockade of α–adrenergic receptors during hypoxia results in no increase in peripheral resistance, while heart rate and cardiac output are increased rather than depressed (Reuss *et al.*, 1982; Jones and Ritchie, 1983). Elevated arterial pressure stimulates arterial baroreceptors, resulting in the reflexly slowed heart rate and reduced cardiac output seen during fetal hypoxia (Thornburg, 1991).

A functional autonomic nervous system is not required for the fetal hypoxic response to occur, as this response is seen prior to the development of functional adrenal innervation (Comline and Silver, 1961; Seidler and Slotkin, 1985; Seidler and Slotkin, 1986b; Seidler and Slotkin, 1986a; Slotkin and Seidler, 1988; Cheung, 1990; Adams *et al.*, 1996). After the establishment of adrenal innervation, this non-neurogenic response to hypoxia is suppressed by factors thought to be released from these nerves and the non-neurogenic response is replaced by the reflex mechanism which exists in the mature animal (Comline and Silver, 1961; Slotkin and Seidler, 1988; Cheung, 1990; Thompson *et al.*, 1997). This suppression of the non-neurogenic response to hypoxia will be discussed in more detail in Section 1.4 and 4.

1.2.2 Carotid body

The carotid body is the primary sensor for detecting changes in plasma O_2 levels and removal of this organ results in the attenuation of ventilatory and cardiovascular responses to hypoxia in humans and newborn lambs (Nakayama, 1961; Bureau *et al.*, 1985). Along with the advent of new technologies such as patch clamping and confocal microscopy came new investigations into how the carotid body can respond to low P_{O_2} levels. The glomus cells, also known as type I cells, of the carotid body had been identified as those responsible for triggering the hypoxic response and it was in glomus cells of the rabbit that K_{O_2} currents were first discovered (Lopez-Barneo *et al.*, 1988). This study found that low P_{O_2} inhibits a voltage-dependent, delayed-rectifier type K^+ channel which is not affected by cellular levels of ATP. The decrease in channel activity is due to decreased open probability rather than changes in single-channel conductance (Ganfornina and Lopez-Barneo, 1991) and this drop in open probability depolarises the membrane, causing Ca^{2+} entry into the cytosol via opening of VGCCs (Buckler and Vaughan-Jones, 1994; Urena *et al.*, 1994). The carotid body is sensitive to decreases in arterial P_{O_2} within the physiological range, as cellular responses to hypoxia such as Ca^{2+} entry and membrane depolarisation increase in a linear fashion as P_{O_2} drops from 150 to 70 mmHg (Ganfornina and Lopez-Barneo, 1991; Duchen and Biscoe, 1992).

During acute hypoxia, neurotransmitters including dopamine (Urena *et al.*, 1994), substance P (Kim *et al.*, 2001) and acetylcholine (Fitzgerald *et al.*, 2000) are released from glomus cells (Urena *et al.*, 1994) and are thought to increase the discharge of the carotid sinus nerve (Gonzalez *et al.*, 1977; Buckler and Vaughan-Jones, 1994; Fitzgerald and Shirahata, 1994; Gonzalez *et al.*, 1994; Kim *et al.*, 2001). Hypoxia has been firmly established as causing dopamine release from the carotid body by Ca^{2+}-dependent means (Fidone *et al.*, 1988). While catecholamine release and sinus nerve activity both increase

during hypoxia, repeated hypoxic bouts show the level of dopamine release declining to a greater degree than nervous activity (Donnelly, 1995). This fall in dopamine release during repeated hypoxic episodes could be due to G protein-mediated inhibition of Ca^{2+} channels by dopamine in these cells, causing negative-feedback inhibition of dopamine release (Benot and Lopez-Barneo, 1990). However, pretreatment with reserpine, which blocks the packaging of catecholamine vesicles, nearly abolishes catecholamine release during hypoxia but does not effect the normal carotid sinus nerve response to hypoxia (Donnelly, 1995), casting doubt as to whether dopamine is the neurotransmitter released from glomus cells which causes increased afferent nerve activity.

Substance P (SP) is also released from the rabbit carotid body by hypoxia in a Ca^{2+}-dependent manner involving L- and N-type Ca^{2+} channels, and SP-like immunoreactivity is localised to glomus cells and surrounding nerve fibres (Kim *et al.*, 2001). SP antagonists also block the sensory response to hypoxia, but not to hypercapnia (Prabhakar, 1994). Acetylcholine has also been shown to be released from the cat carotid body during hypoxia (Fitzgerald *et al.*, 2000) and this biogenic amine has a stimulatory effect on cat, and inhibitory effect on rabbit carotid bodies (Prabhakar, 2000). The sensory response to hypoxia, however, is only partially blocked by high concentrations of muscarinic and nicotinic antagonists applied simultaneously in cat carotid bodies (Fitzgerald *et al.*, 1999). While most attention has been placed on the release of dopamine during hypoxia and the role it plays as the sensory neurotransmitter of the carotid body, it now seems clear that other neurochemicals including SP and acetylcholine also play a role in the control of sensory discharge from this tissue during hypoxia.

In whole cell recordings from rabbit glomus cells, hypoxia increases the frequency of AP firing and reduces the K_{O_2} current by a mechanism described as being independent of internal Ca^{2+} or ATP (Lopez-Lopez *et al.*, 1989). Of the three different types of K^+

current described in these cells, only the amplitude of a fast-inactivating, voltage-dependent, Ca^{2+}-insensitive K^+ current, is reduced by hypoxia (Ganfornina and Lopez-Barneo, 1992). The identity of the K_{O_2} channel, however, has remained unknown for many years, as the structural intricacy and small size of this organ makes the K_{O_2} channels difficult to characterise with most molecular biological techniques. The infection of rabbit carotid bodies with adenoviruses expressing green fluorescent protein with a dominant-negative construct for the Kv4 channel subfamily, results in almost complete blockade of the K_{O_2} current (Perez-Garcia *et al.*, 2000). The blockade of O_2-sensitive current by this construct depolarises the cells and suppresses hypoxia-induced membrane depolarisation (Perez-Garcia *et al.*, 2000), suggesting a role for this channel in controlling the resting membrane potential and initiating hypoxia-evoked chemotransduction. Very recently, investigators have identified Kv4.1 and Kv4.3 as the molecular correlates of this O_2-sensitive, fast-inactivating current in rabbit glomus cells (Sanchez *et al.*, 2002).

Much of the work on identifying possible K_{O_2} channels in the carotid body has used the rat as a model mammalian system. One channel which has been identified as O_2-sensitive in rat glomus cells is the BK channel. Early work on this tissue found that K^+ current suppression by hypoxia is abolished upon Ca^{2+} channel blockade (Peers, 1990), and a $K_{(Ca)}$ channel is responsible for the hypoxic suppression of K^+ current seen in glomus cells in 4-day-old, 10-day-old and adult rats (Hatton *et al.*, 1997). Glomus cells from rats reared in a chronically hypoxic (10% O_2) environment still display hypoxic suppression of K^+ currents but do not depolarise during acute hypoxia or contain any charybdotoxin-sensitive K^+ current, as do normal glomus cells (Wyatt *et al.*, 1995). The lack of a charybdotoxin-sensitive K^+ current in chronically hypoxic rats was attributed as being responsible for this deficit in hypoxia-induced depolarisation and hence, BK channels were proposed as the O_2-sensitive $K_{(Ca)}$ channel in glomus cells. This was confirmed recently

when BK currents in rat glomus cells were shown to be inhibited by hypoxia in both the whole cell and inside-out patch clamp configuration (Riesco-Fagundo *et al.*, 2001). This effect is strongly voltage- and Ca^{2+}-dependent, being maximal at low [Ca^{2+}] and more negative membrane potentials.

The exact role which these BK channels play in the chemotransduction of carotid bodies remains controversial due to the variable effects of BK channels antagonists. TEA and charybdotoxin do not elicit secretion from whole carotid body preparations (Osanai *et al.*, 1997; Lahiri *et al.*, 1998) and while TEA does not increase $[Ca^{2+}]_i$ or depolarise the plasma membrane (Buckler, 1997), charybdotoxin has been shown to depolarise the membrane of isolated glomus cells (Wyatt and Peers, 1995). Hypoxia and the BK channel antagonist, iberiotoxin, both evoke similar degrees of catecholamine release from rat carotid body slices (Pardal *et al.*, 2000). Whether BK channels are responsible for this hypoxia-evoked secretion is unclear, however, as the effect of both hypoxia and iberiotoxin together was not investigated.

The resting membrane potential for isolated glomus cells has been reported to be between -60 mV to -43 mV (Buckler and Vaughan-Jones, 1994; Wyatt *et al.*, 1995; Buckler, 1997) and BK channels have been reported as requiring voltages above those at the resting membrane potential of glomus cells to activate their opening (Buckler, 1997; Salapatek *et al.*, 2002). This fact, combined with the variable effects of BK channel antagonists on carotid body activity, make the involvement of BK channels in instigating the hypoxic secretory response from the carotid body questionable.

A K_{O_2} current in rat glomus cells which is not voltage, TEA-, or 4-AP-sensitive, as has been previously described, has also been uncovered and this current is termed the background leak K^+ current (Buckler, 1997). This current is inhibited by Ba^{2+} which causes a membrane depolarisation, similar to that caused by hypoxia, of approximately 15-20 mV

(Buckler, 1999). The leak current has a single channel conductance of 14 pS, is inhibited not only by Ba^{2+} but also by zinc, bupivacaine and quinidine, is pH-sensitive and is stimulated by halothane, but not chloroform (Buckler *et al.*, 2000). These pharmacological and biophysical properties of this leak K^+ channel are compatible with it being a TASK-1 channel and *in situ* hybridisation has illustrated that TASK-1 mRNA is present in rat glomus cells (Buckler *et al.*, 2000). TASK-1 channels consist of four membrane-spanning and two pore-forming domains, but lack the charged, voltage-sensing region of many K^+ channels making gating independent of the membrane potential (Gurney *et al.*, 2002).

It therefore appears that two types of K_{O_2} channels exist in the rat carotid body. One of them, the acid-sensitive TASK-1 channel, is capable of triggering the cell response during hypoxia, as it is open at resting membrane potentials and its closure causes significant membrane depolarisation. The other, a $K_{(Ca)}$ channel which is most likely the BK channel, still has a disputable role in initiating the hypoxic response but is responsible for some hypoxic suppression of K^+ current and seems likely to play at least a modulatory role in the hypoxic response. The identification of these two channels as the K_{O_2} channels in the rat carotid body is further endorsed by recent findings which show that recombinantly expressed BK (Lewis *et al.*, 2002) and TASK-1 (Lewis *et al.*, 2001) channels are both sensitive to hypoxia.

1.2.3 Neuroepithelial bodies

Neuroepithelial bodies (NEB) are clusters of neurone-derived cells, with which both afferent and efferent neurone branches synapse, that are widely dispersed throughout the airway epithelium of mammals. NEB play a role in matching the regional ventilation of the lungs to the perfusion of that area. During instances when the P_{O_2} of the inspired air

decreases, serotonin is released by the NEB (Lauweryns and Cokelaere, 1973) which gives rise to local vasoconstriction in the hypoxic lung areas, shunting blood from inadequately ventilated to better ventilated parts of the lung (Cutz and Jackson, 1999).

P_{O_2} levels less than or equal to 25-30 mmHg cause K^+ channel inhibition (Youngson *et al.*, 1993), membrane depolarisation (O'Kelly *et al.*, 1998) and activation of VGCCs in NEB, triggering serotonin release (Fu *et al.*, 2002). Similar to glomus cells, Ca^{2+}-dependent, voltage-dependent and voltage-independent K^+ channels are all O_2-sensitive in NEB of isolated (Youngson *et al.*, 1993) and *in situ* (Fu *et al.*, 1999) preparations. In lung slice preparations of rabbit neonates, hypoxia (P_{O_2} = 15-20 mmHg) reduces both Ca^{2+}- and voltage-dependent components of the outward K^+ current equally, an effect suppressed in the presence of either TEA or 4-AP, respectively (Fu *et al.*, 1999). The K_{O_2} channel in NEB has been proposed as voltage-gated Kv3.3 channels, as the K_{O_2} current in these cells has similar inactivation properties to that of Kv3.3 channels expressed in *Xenopus laevis* oocytes (Vega-Saenz de Miera and Rudy, 1992) and Kv3.3α mRNA has been identified in fetal rabbit and neonatal human lungs (Wang *et al.*, 1996). Whether these channels are capable of initiating the hypoxia-induced membrane depolarisation in these cells is disputable however, as Kv3.3 currents activate at voltages positive to -20 mV (Rashid *et al.*, 2001), implying that these channels will be closed at resting membrane potential. The voltage-independent (Youngson *et al.*, 1993) and Ca^{2+}-dependent (Fu *et al.*, 1999) components of K_{O_2} currents in NEB, are yet to be identified.

NEB represent less than 1% of epithelial cells in human lungs (Cutz *et al.*, 1984) and because of this and its widespread distribution in such a large organ, culturing NEB cells is difficult. To circumvent this problem, the small cell lung carcinoma cell line, H-146, derived from NEB, has been utilised as a model system for studying O_2 sensing mechanisms in human NEB cells. The K^+ current suppressed by hypoxia (P_{O_2} = 15-20

mmHg) in these model cells is Ca^{2+}-independent, and hypoxia and TEA both depolarise the membrane with additive effect (O'Kelly *et al.*, 1998), indicating that the K_{O_2} channel in these cells is TEA-insensitive. It is possible that this K_{O_2} current is the same as that in native NEB cells, as both currents have similar pharmacology and biophysical properties (Peers and Kemp, 2001). Systematic pharmacological characterisation of the K_{O_2} current found it to be TEA- and dithiothreitol (DTT)-insensitive, pH-dependent, blocked by arachidonic acid and activated by halothane (Hartness *et al.*, 2001). These properties, and the discovery of mRNA for both hTASK1 and hTASK3 in H-146 cells (O'Kelly *et al.*, 1999; Hartness *et al.*, 2001), identified these two channels as the possible K_{O_2} channels in H-146 cells, a fact confirmed when the use of antisense oligonucleotides directed against hTASK1 and hTASK3 eliminated the hypoxic response in these cells (Hartness *et al.*, 2001). The final discriminative tool for the identification of the O_2-sensitive channel in H-146 cells, utilised varying concentration-dependent blocking of hTASK channels with Zn^{2+}. In the presence of a Zn^{2+} concentration which blocks hTASK-1, but not hTASK-3, the hypoxic suppression of K^+ current remains, identifying hTASK3 as the K_{O_2} channel in H-146 cells (Hartness *et al.*, 2001).

It appears that in NEB, as in carotid body glomus cells, more than one type of K_{O_2} channel may exist. While the Kv3.3 has been postulated as O_2-sensitive in native NEB cells, this channel has not been shown to influence resting membrane potential. The hTASK3 channel is capable of altering the resting membrane potential and while it remains to be seen if hTASK3 is a functional K_{O_2} channel in native NEB cells, it is conceivable that these two channel types may work in conjunction with each other to mediate NEB responses to hypoxia.

1.2.4 Pulmonary smooth muscle

In areas of the lung where alveolar O_2 levels are low, localised pulmonary artery vasoconstriction occurs. This mechanism of vasoconstriction, in conjunction with the actions of NEB during hypoxia, diverts blood flow away from these hypoxic regions to better ventilated areas of the lung where gas exchange can take place more effectively. This phenomenon is known as hypoxic pulmonary vasoconstriction (HPV) and is unique to pulmonary circulation, as hypoxia produces vasodilation in systemic arteries. HPV is caused by the contraction of pulmonary artery smooth muscle cells (PASMC) produced via a hypoxic response mechanism similar to that found in neurosecretory O_2-sensitive cells. PASMC contain K_{O_2} channels which close during hypoxia, causing membrane depolarisation (Harder *et al.*, 1985; Madden *et al.*, 1985), Ca^{2+} entry into the cytosol, cell contraction (Salvaterra and Goldman, 1993; Gelband and Gelband, 1997) and a resulting vasoconstriction. These cells respond to P_{O_2} levels of 15-20 mmHg in dissected arteries (Leach *et al.*, 2001) and the isolated perfused rat lung (Archer *et al.*, 1993). Chronic hypoxia in adults causes a sustained HPV leading to vascular remodeling, pulmonary hypertension and possible right heart failure and death (Sweeney and Yuan, 2000).

The identity of the K_{O_2} channel(s) in PASMC remains a point of ongoing study. At rest, the membrane potential of PASMC is around -50 mV (Casteels *et al.*, 1977) and it has been postulated that a voltage-dependent, delayed rectifying K^+ channel maintains this membrane potential, not a Ca^{2+}-activated or inwardly rectifying K^+ channel (Yuan, 1995; Archer *et al.*, 2000). Hypoxia inhibits three voltage-gated, 4-AP-sensitive channels expressed in PASMC, namely Kv1.2, Kv2.1 and Kv3.1 (Hulme *et al.*, 1999; Osipenko *et al.*, 2000). There is, however, doubt as to whether these channels account for hypoxia-induced membrane depolarisation in these cells as they have activation thresholds around 40 mV (Coetzee *et al.*, 1999). The sensitivity of Kv2.1 and Kv1.2 channels to hypoxia is

increased when they form heterologous channels with Kv9.3 and Kv1.5 α subunits, respectively (Patel *et al.*, 1997; Hulme *et al.*, 1999), and these heterologous channels activate at more negative potentials, making their contribution to resting membrane potential more likely. Whether these heterologous channels actually form in native PASMC remains to be seen.

Another possible K_{O_2} channel in PASMC produces a low threshold, non-inactivating K^+ current, named I_{KN} (Osipenko *et al.*, 1997), which is inhibited by hypoxia and mediates the hypoxia-induced depolarisation in rabbit PASMC (Osipenko *et al.*, 1998). Channel gating is different from the delayed rectifier current, with a threshold for channel opening at -70 mV and different activation kinetics from delayed rectifiers (Evans *et al.*, 1996) making it unlikely that the heteromeric Kv2.1/Kv9.2 channel accounts for this I_{KN} current. The pharmacological properties of TASK-like currents have several features in common with I_{KN} currents in PASMC, including low sensitivity to TEA, quinine, glibenclamide and Ba^{2+}, and a lack of sensitivity to Ca^{2+} or ATP (Evans *et al.*, 1994; Evans *et al.*, 1996; Osipenko *et al.*, 1997). The major common feature of these two current types is their high sensitivity to pH and the possibility that a TASK channel may underlie the I_{KN} current was strengthened when rabbit PASMC were found to stain positively with antibodies for TASK-1 channel proteins (Gurney *et al.*, 2002).

It appears that in PASMC, several types of delayed rectifier Kv currents are suppressed by hypoxia, as are TASK-1 currents. As the TASK-1 channels, unlike Kv channels, are open at resting membrane potential, this K_{O_2} channel seems most likely to initiate HPV in pulmonary arteries.

1.2.5 PC-12 cells

The PC-12 cell line is derived from a rat pheochromocytoma, a tumor of AMCCs. These cells have been traditionally used as a model system of cellular O_2 sensing for many O_2-sensitive cells. PC-12 cells have many phenotypic similarities with glomus cells and AMCCs, such as the existence of K_{O_2} channels (Lopez-Barneo *et al.*, 1988; Conforti and Millhorn, 1997; Lee *et al.*, 2000), and the induction by hypoxia of tyrosine hydroxylase (TH) gene expression upregulation (Czyzyk-Krzeska *et al.*, 1992; Czyzyk-Krzeska *et al.*, 1994; Mamet *et al.*, 2002), membrane depolarisation and catecholamine secretion (Urena *et al.*, 1994; Inoue *et al.*, 1998; Kumar *et al.*, 1998; Taylor and Peers, 1998; Buckler *et al.*, 2000).

Hypoxia (P_{O_2} = 11 mmHg), causes Ca^{2+} influx through N-type Ca^{2+} channels which stimulates catecholamine secretion from single PC-12 cells through a mechanism which is TEA-sensitive and 4-AP-insensitive (Taylor and Peers, 1998). This is in agreement with earlier findings describing the O_2-sensitive current as a slowly inactivating, TEA-sensitive and Ca^{2+}-insensitive K^+ current, which causes a membrane depolarisation of 8 mV and a two-fold increase in $[Ca^{2+}]_i$ during hypoxia (Zhu *et al.*, 1996). A slow-inactivating 20 pS K^+ channel is responsible for the only K^+ current present in PC-12 cells which is O_2-sensitive, and genes encoding for the α-subunit of the slow-inactivating Kv1.2 channel have been identified in PC-12 cells, with the gene expression for this channel increasing upon exposure to hypoxia for 18 hours (Conforti and Millhorn, 1997).

This Kv1.2 channel was subsequently expressed in *Xenopus laevis* oocytes and the K^+ currents created by this channel were found to be O_2-sensitive (Conforti and Millhorn, 2000). Antibodies specific for the Kv1.2 channel dialysed through the patch pipette completely block this K_{O_2} current in PC-12 cells (Conforti *et al.*, 2000). These results

strongly indicate that the Kv1.2 channel plays a critical role in the response of PC-12 cells to hypoxia.

1.2.6 O_2-sensitive Ca^{2+} channels

Recent investigations suggest that some Ca^{2+} channels are also capable of being directly influenced by changes in O_2 tension. In freshly dissociated rabbit glomus cells, hypoxia (P_{O_2} = 40 mmHg) augments L-type Ca^{2+} current by approximately 25% in a voltage-independent manner via the activation of a PKC-sensitive mechanism (Summers *et al.*, 2000). L-type current is also potentiated by decreased O_2 tension in inspiratory neurones of neonatal mice by increasing the open probability of these channels, indicating that these channels have a role in the early hypoxic response of the respiratory centre (Mironov and Richter, 1998). In coeliac and femoral arterial myocytes, hypoxia (P_{O_2} = 20 mmHg) inhibits L-type, but not T-type, Ca^{2+} channels in a voltage-dependent manner, suggesting L-type Ca^{2+} channels have a localised function in regulating circulation (Franco-Obregon *et al.*, 1995). This contribution by Ca^{2+} channels to the regulation of local circulation was made more evident when smooth muscle cells from conduit (proximal) and resistance (distal) rabbit pulmonary arteries were found to respond differently to hypoxia through inhibition and potentiation of Ca^{2+} currents, respectively (Franco-Obregon and Lopez-Barneo, 1996). This may help explain how hypoxia causes vasodilation in conduit arteries and vasoconstriction in resistance vessels.

The O_2-sensitive Ca^{2+} channels in native cells are most often L-type channels, and recombinant human cardiac L-type Ca^{2+} channel α1C subunits expressed in HEK 293 cells are also affected by hypoxia, with a drop in P_{O_2} inhibiting these channels (Fearon *et al.*, 1999). Somewhat surprisingly, the oxidising agent p-chloromercuribenzene sulphonic acid

(PCMBS), but not the reducing agent DTT, also inhibit these recombinant channels. Hypoxia is generally thought to modulate channels by reducing the redox state of cells (discussed in Section 1.3). In the presence of PCMBS, hypoxia has no effect on Ca^{2+} current amplitude and distinct cysteine residues on the α1C subunit appear likely to undergo redox modulation during hypoxia (Fearon *et al.*, 1999). Further work on this topic found that hypoxia inhibits only one of the three naturally occurring splice variants of this channel that differ only in the C-terminal domain, and selective deletion of this region identified a 39-amino acid region essential for O_2 sensing (Fearon *et al.*, 2000b).

Currents from the stably expressed T-type channel $\alpha_1$3.2 and $\alpha_1$3.3 subunits, but not the $\alpha_1$3.1 subunit, are inhibited by hypoxia in a non voltage-dependent manner, with the effect on the $\alpha_1$3.2 currents being more marked than on the $\alpha_1$3.3 currents (Fearon *et al.*, 2000a). Similarly to recombinant L-type Ca^{2+} currents, currents from the $\alpha_1$3.2 and $\alpha_1$3.3 channels are effected by changes in the intracellular redox state, with the reducing agent GSH causing a decrease in current and the oxidant GSSG increasing current amplitude (Fearon *et al.*, 2000a). The hypoxic suppression of $\alpha_1$3.2 current, however, is independent of changes in the level of the intracellular redox couple GSH:GSSG (Fearon *et al.*, 2000a), indicating that hypoxic and redox modulation of this T-type Ca^{2+} channel occur through different mechanisms.

1.2.7 O_2-sensitive Na^+ channels

Considerable research has been undertaken on the subject of hypoxic sensitivity of voltage-gated Na^+ channels, particularly in areas of the central nervous system where cells are extremely vulnerable to hypoxic injury during episodes of stroke or apnoea (Fung, 2000). The blockade of voltage-gated Na^+ channels reduces the neuronal response and

injury generated by hypoxia (Urenjak and Obrenovitch, 1996). Hypoxia appears to have differential effects on particular voltage-gated Na^+ channel types, as it rapidly inhibits the transient Na^+ current in hippocampal CA1 neurones (Cummins *et al.*, 1993), while in the same cell type the persistent Na^+ current is increased upon hypoxia or cyanide exposure (Hammarstrom and Gage, 1998; Hammarstrom and Gage, 2000). The activity of this persistent Na^+ current is similarly escalated during hypoxia in cardiac myocytes due to an increased open probability of these channels (Ju *et al.*, 1996). Chronic hypoxia produces significant increases in Na^+ current density within a few days through a cAMP-mediated pathway in dissociated rat carotid body glomus cells, and this may underlie the increased sensitivity in the acute hypoxic response of these cells (Stea *et al.*, 1992).

Blockade of Na^+ channels in AMCCs does not alter the resting membrane potential or prevent these cells from firing AP (Hollins and Ikeda, 1996), and this may be why the topic of Na^+ current modulation by hypoxia has not been investigated in AMCCs. Although it is difficult to perceive how changes in Na^+ channel opening by acute hypoxia would effect changes in hypoxia-induced Ca^{2+} entry and catecholamine secretion, chronic hypoxia in AMCCs may significantly alter Na^+ channel expression, as it does in glomus cells (Stea *et al.*, 1992), causing substantial changes in the way AMCCs respond to subsequent hypoxic episodes.

1.3 Intracellular mechanisms of oxygen sensing

Several theories have been proposed to explain how decreased environmental P_{O_2} levels induce changes in ion channel function in O_2-sensitive cells. Variations in the production of reactive oxygen species (ROS) within the cytoplasm are popularly believed to cause these alterations in channel function, and the main intracellular systems involved

in the production of ROS, mitochondria and NADPH oxidase, have been proposed as regulators of ROS levels during hypoxia. There are also some K_{O_2} channels which seem to have no reliance on intracellular components and respond to P_{O_2} changes directly. The following section will review different O_2 sensing mechanisms across a variety of O_2-sensitive cell types.

In the mitochondria, around 1-2% of total O_2 consumption results in the production of the potentially cytotoxic ROS, superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), during the reduction of O_2 to water in the electron transport chain (ETC) (Chance *et al.*, 1979). O_2^- can be either spontaneously or enzymatically converted to H_2O_2 at extremely high rates and this mitochondrial H_2O_2 is eliminated in this organelle by glutathione peroxidase. Despite this, significant amounts of H_2O_2 diffuse from the mitochondria into the cytosol (Chance *et al.*, 1979; Turrens and Boveris, 1980; Turrens *et al.*, 1985). NADPH oxidase is a membrane bound multi-subunit enzyme system which indirectly produces H_2O_2 by reducing O_2 to O_2^- (Acker, 1994) which is then converted either spontaneously or enzymatically in the cytosol to H_2O_2.

The contribution of ROS in O_2 sensing was first considered upon the discovery that rat carotid bodies produce H_2O_2 and that H_2O_2 scavengers cause a decrease in chemoreceptor discharge (Acker *et al.*, 1992). Around the same time, three different cloned voltage-gated K^+ channels expressed in *Xenopus laevis* oocytes were found to have their rate of time-dependent inactivation altered by external H_2O_2 application, illustrating that ROS levels can alter ion channel function (Vega-Saenz de Miera and Rudy, 1992).

In O_2 sensing cells, heme-containing proteins, including those in NADPH oxidase and mitochondria, are thought to interact with H_2O_2 and produce the highly reactive hydroxyl ion (OH^-) through the Fenton reaction (shown below).

Fenton Reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \cdot OH$$

Both H_2O_2 and OH^- can cause site specific oxidation of proteins such as those containing thiol groups, and if these groups are situated within areas which effect ion channel structure or function, then changes in local redox state during hypoxia could conceivably cause changes in channel gating.

1.3.1 Membrane delimited oxygen sensing

Many proposed O_2 sensing mechanisms have connected the modification of K^+ channel function to various intracellular factors. However, some K_{O_2} channels respond to hypoxia in a membrane delimited fashion, independent of the intracellular conditions. This was first illustrated in rabbit carotid body glomus cells where a K^+ channel with a single channel conductance of 20 pS is modulated by changes in P_{O_2} in excised membrane patches (Ganfornina and Lopez-Barneo, 1991). BK channels excised in the inside-out configuration are also inhibited by hypoxia in rat glomus cells due to an increased time spent in the longest closed state when P_{O_2} levels are reduced (Riesco-Fagundo *et al.*, 2001). The reducing agent DTT increases BK channel open probability in these cells, whereas oxidising agents have the opposite effect, suggesting that hypoxic inhibition of this channel is not related to a reduction of channel thiol groups (Riesco-Fagundo *et al.*, 2001).

Some neurons which respond to hypoxia via the actions of K_{O_2} channels are also capable of doing so through a membrane-delimited mechanism. In dissociated cells from

the rat neocortex and substantia nigra, hypoxia (P_{O_2} = 10 mmHg) reversibly inhibits a large conductance, ATP- and Ca^{2+}-sensitive K^+ channel by approximately 50% in excised membrane patches (Jiang and Haddad, 1994). Cultured hippocampal neurons contain an O_2-sensitive persistent Na^+ current which is activated by hypoxia in both cell-attached and inside-out patch clamp configurations (Hammarstrom and Gage, 2000). This effect of hypoxia on inside-out patches is replicated by the application of both DTT and reduced glutathione (GSH), suggesting the involvement of redox state in this hypoxic activation of current (Hammarstrom and Gage, 2000).

Some heterologously expressed channels also retain O_2 sensitivity in excised membrane patches. Recombinant human brain BK channels, expressed in HEK 293 cells, are reversibly suppressed by hypoxia (P_{O_2} = 40 mmHg) in inside-out patches (Lewis *et al.*, 2002). Kv3.1b, but not Kv1.2 or Kv1.5 channels, expressed in L929 cells, show hypoxic inhibition in excised patches (Osipenko *et al.*, 2000) and the Kv1.2 β-subunit confers sensitivity to hypoxia and redox modulation in excised patches containing Kv4.2, but not *Shaker*, channel α-subunits expressed in HEK 293 cells (Perez-Garcia *et al.*, 1999). G protein-coupled inwardly rectifying K^+ channels, expressed in *Xenopus laevis* oocytes, are activated by DTT but not GSH in a membrane delimited fashion due to changes in a critical cysteine residue located in the N-terminal cytoplasmic domain (Zeidner *et al.*, 2001).

While some K_{O_2} currents remain responsive to hypoxia in excised membrane patches, this is not always the case. The TASK-1 channel, identified as a likely K_{O_2} channel in rat glomus cells, loses hypoxic inhibition upon excision of the patch in the inside-out configuration (Buckler *et al.*, 2000). Similarly, the O_2-sensitive BK current in rat glomus cells is significantly reduced by hypoxia and anoxia in perforated, but not outside-

out patches (Wyatt *et al.*, 1995), indicating the O_2-dependence of this channel is mediated by cytosolic factors.

It appears that these effects of hypoxia and reducing agents on excised membrane patches illustrate that cytosolic components are not always required for ion channel O_2 sensing. Currently, no ion channel is known to contain a putative O_2 sensing component, such as a heme protein, making it difficult to comprehend how ion channels would be capable of responding to alterations in P_{O_2} levels directly. The presumption that excised patches function in a manner which is independent of cytosolic factors may not be true, as recent evidence shows that mitochondria are attached to excised patches in pancreatic β-cells (Rustenbeck *et al.*, 1999). Further evidence will be required to find whether these or other organelles are attached to membrane patches containing O_2-sensitive channels.

1.3.2 NADPH oxidase

NADPH oxidase is a multisubunit system consisting of the membrane-bound catalytic $gp91^{phox}$ and $p22^{phox}$ subunits, together with the b_{558} flavo-cytochrome and a regulatory component within the cytosol consisting of $p47^{phox}$ and $p67^{phox}$ subunits (Youngson *et al.*, 1997; Chandel and Schumacker, 2000). In neutrophils, NADPH oxidase activation is regulated by the cytosolic components which relocate to the membrane and associate with cytochrome b_{558} to initiate catalytic activity (Cross *et al.*, 1999). It is hypothesised that, during hypoxia, decreased O_2 availability leads to a reduced level of electron transport through the NADPH oxidase system, slowing the rate of ROS production, shifting the cytosol to a more reduced redox state and altering channel structure and function.

The involvement of NADPH oxidase in O_2 sensing was first inferred in the rat carotid body when it was found that hypoxia produces an optical absorbance spectrum in this tissue resembling that of reduced NADPH oxidase in neutrophils, and that this effect is attenuated by the NADPH oxidase antagonist, diphenyliodonium (DPI, also known as diphenylene iodonium) (Acker *et al.*, 1989). A *b*-type cytochrome was identified in this tissue through photometric measurements as that responsible for producing this change in absorbance spectrum, and H_2O_2 formation and hypoxia-induced chemoreceptor discharge were both inhibited in the presence of DPI (Cross *et al.*, 1990). DPI similarly inhibits hypoxic pulmonary vasoconstriction in perfused rat lungs (Thomas *et al.*, 1991), raising the possibility that this mechanism of O_2 sensing may not be confined to the carotid body. This possibility was reinforced when O_2-sensitive fetal rabbit cultured NEB were found to express NADPH oxidase in their plasma membrane (Youngson *et al.*, 1993).

The importance of NADPH oxidase in all O_2 sensitive cells may have been overestimated, however, as recent studies provide strong evidence that inhibition of NADPH oxidase does not interfere with low P_{O_2} transduction in the carotid body or PASMC. In mice where the DNA sequence of $gp91^{phox}$ has been disrupted, whole glomus cell K^+ current amplitude and the effect of hypoxia on K^+ current depression, $[Ca^{2+}]_i$ increases, whole animal ventilatory responses, and carotid sinus nerve activity, are all indistinguishable from those of wild-type mice (Roy *et al.*, 2000; He *et al.*, 2002). In PASMC of NADPH oxidase-deficient mice, a severe reduction in ROS production does occur but the hypoxic inhibition of whole cell K^+ currents (in PASMCs) and HPV (in isolated lungs) is identical to that of wild-type mice (Archer *et al.*, 1999). It therefore appears that, although NADPH oxidase is a major source of lung ROS production in mice PASMCs, it is not the O_2 sensor in these cells.

While DPI causes a concentration-dependent release of catecholamines from both rat and rabbit glomus cells, it does not alter secretion levels due to hypoxia, as stimulation of these cells with both hypoxia and DPI elicited a response equal to the sum of either hypoxia or DPI alone (Obeso *et al.*, 1999). Other inhibitors of NADPH oxidase (neopterin and phenylarsine oxide) do not trigger secretion during normoxia nor effect the hypoxic secretory response (Obeso *et al.*, 1999). This difference in the effect of DPI and other NADPH oxidase inhibitors may be due to functional inhibition of multiple flavoproteins caused by DPI application (O'Donnell *et al.*, 1994). Also, DPI causes non-selective inhibition of ion channels in glomus cells (Weir *et al.*, 1994) and PASMC (Weir *et al.*, 1994), and this inhibition of ion channels could explain the secretion or vasoconstriction which is seen in these cells during DPI application.

In NEB, however, NADPH oxidase does appear to have a role in O_2 sensing. The mRNA for the NADPH oxidase subunits, $gp91^{phox}$ and $p22^{phox}$, is expressed in NEB cells of fetal rabbit and neonatal human lungs, as well as in H-146 cells (Wang *et al.*, 1996). In cultured fetal and neonatal rabbit NEB, the hypoxic suppression of K^+ current is inhibited by DPI (Wang *et al.*, 1996; Fu *et al.*, 1999) and most importantly, this suppression of hypoxic K^+ current inhibition by DPI is seen in NEB of wild-type, but not NADPH oxidase-deficient gene knock-out mice (Fu *et al.*, 2000). NADPH oxidase may not be the sole O_2 sensor in H-146 cells, however, as DPI and phenylarsine oxide both suppress, but do not fully block, the inhibition of K^+ currents during hypoxia (O'Kelly *et al.*, 2001), indicating that multiple O_2 sensing mechanisms may exist in this cell line.

1.3.3 Mitochondria

In the mitochondria, a system of electron transport exists, involving five enzyme complexes named complex I, II, III, IV and V, and this system is known as the electron transport chain (ETC). Complex I, III and IV are all proton pumps which use the energy of electron transfers to translocate protons from the mitochondrial matrix to the mitochondrial cytosol in order to sustain an electrochemical gradient across the inner mitochondrial membrane (Schagger, 2001). Complex V then uses the free energy released from the flow of protons back across the inner membrane to perform the chemical work required to phosphorylate ADP and produce ATP (Schultz and Chan, 2001). Complex I oxidises NADH to NAD^+ and transfers an electron to ubiquinol while complex II oxidises succinate to fumarate and also transfers an electron to ubiquinol (Cadenas and Davies, 2000). Ubiquinol is a two electron reductant which is oxidised in one-electron steps. This makes it possible for ubiquinol to accept two electrons at these two different enzyme complexes and these electrons are then transferred to ferricytochrome c in complex III (Schultz and Chan, 2001). The terminal electron acceptor in this chain is located at complex IV where electrons are transferred from ferrocytochrome c to O_2, thus oxidising cytochrome c back to the ferric form and reducing O_2 into water in the reaction shown below.

$$O_2 + 4e^- + 4H^+ \rightarrow 2H_2O$$

The major role of O_2 in the ETC is, therefore, to act as an acceptor of electrons from cytochrome oxidase. While this system of electron transport is very efficient, the one-electron reactions which occur throughout this cycle are susceptible to side reactions with molecular O_2. The classical example of this is the partially reduced form of ubiquinol, semiubiquinone, which has an electron that can be freely donated to O_2 to form O_2^- before

the electron reaches the next electron carrier in the chain (Droge, 2002). Thus, instead of being converted to water, approximately 1-2% of mitochondrial O_2 consumption goes towards O_2^- formation.

As mitochondrial electron transport is terminated in the complete absence of O_2, the concept that ROS levels maintained by mitochondria would be significantly decreased during anoxia is a simple one. How the mitochondria could affect intracellular changes when P_{O_2} levels alter within the physiological range is, however, more difficult to comprehend. Cellular respiration is not limited by O_2 supply until extra-mitochondrial P_{O_2} falls below 5-7 mmHg due to the low K_m value for oxygen of several mitochondrial enzyme complexes (Jones and Mason, 1978; Wilson *et al.*, 1988). The function of many O_2-sensitive ion channels, however, is altered at P_{O_2} levels far greater than this value. P_{O_2} gradients between the plasma membrane and the mitochondria are thought to exist in mammalian cells (Jones and Mason, 1978; Kennedy and Jones, 1986) and these gradients may explain how mitochondria could detect physiological changes in O_2 levels. Alternatively, tissue-specific isoforms of various ETC complexes could exist with much higher K_m values for oxygen than those previously measured, although experimental evidence of this has not emerged.

In the carotid body, cyanide has long been known to be a potent stimulant (Duchen and Biscoe, 1992b) and this observation adds support to the theory that mitochondria are involved in O_2 sensing, as cyanide disrupts ETC function at complex IV. However, care must be taken when interpreting data which utilises cyanide as an ETC inhibitor. This is because cyanide is capable of causing Ca^{2+} release from intracellular stores (Biscoe *et al.*, 1989) which can increase cytoplasmic $[Ca^{2+}]$ and Ca^{2+}-dependent secretion of neurotransmitters. Therefore, results which state that cyanide mimics the effects of hypoxia by increasing $[Ca^{2+}]_i$ or secretion must be interpreted carefully.

Cyanide also inhibits the activity of cytochrome oxidase, the terminal electron acceptor at complex IV of the ETC. It is conceivable then that the disruption of this oxidase will result in less O_2 conversion to water and an increase in ROS production. During hypoxia the V_{max} for cytochrome oxidase is decreased while the apparent K_m remains unchanged in both cardiomyocytes and hepatocytes (Chandel *et al.*, 1996; Chandel *et al.*, 1997; Budinger *et al.*, 1998). This could result in decreased production of water from O_2, leaving a greater amount of free O_2 available to receive free electrons, increasing the mitochondrial redox state and generating more ROS during hypoxia (Chandel *et al.*, 1995; Chandel *et al.*, 1996). This decrease in the activity of cytochrome oxidase, however, occurs over a time frame of minutes to hours (Chandel *et al.*, 1995; Chandel *et al.*, 1996; Chandel *et al.*, 1997; Budinger *et al.*, 1998), much longer than that required to cause the rapid regulation of O_2-sensitive ion channels.

There is a large pool of evidence which does suggest mitochondrial involvement in O_2 sensing in certain tissues. Several investigations in glomus cells and PASMC have found that a range of mitochondrial inhibitors can reduce K^+ current amplitude or cause membrane depolarisation (Archer *et al.*, 1993; Yuan *et al.*, 1996; Buckler and Vaughan-Jones, 1998; Archer *et al.*, 2000; Michelakis *et al.*, 2002). Antimycin A, which inhibits complex III activity, augments basal secretory activity and prevents the response to hypoxia in the carotid body (Mulligan and Lahiri, 1982). Carbon monoxide (CO) is known to bind with mitochondrial cytochromes, and the involvement of mitochondria in carotid body O_2 sensing would explain why high concentrations of CO reversibly trigger sensory discharge from carotid bodies in a manner similar to hypoxia (Lahiri *et al.*, 1999). The spectral characteristics of this tissue upon CO exposure are those of CO binding to cytochrome oxidase, indicating CO binds with and inhibits complex IV activity (Wilson *et al.*, 1994).

Hypoxia increases NADH autofluorescence in both the carotid body and AMCCs, a response which is indicative of mitochondrial inhibition, and the effects of both hypoxia and cyanide on secretion are not additive, suggesting a common mechanism of action in both the rabbit carotid body and rat neonate AMCCs (Duchen and Biscoe, 1992a; Mojet *et al.*, 1997). Cyanide effects single guinea pig AMCCs in a similar fashion to hypoxia by depolarising the plasma membrane and inducing catecholamine secretion (Inoue *et al.*, 1998). However, this study did not investigate the effect of concurrent application of these two stimuli, making it difficult to interpret whether hypoxia inhibits cytochrome oxidase in these cells. In newborn rat AMCCs, catecholamine secretion and increased NADH autofluorescence only occur once P_{O_2} falls below 5 mmHg, and these hypoxic effects are mimicked by the inhibition of complex I with rotenone (Mojet *et al.*, 1997)

Mitochondria also appear to play a role in O_2 sensing in rat pulmonary arteries by controlling the rate at which HPV occurs. Rotenone-induced vasoconstriction occurs in mice where the $gp91_{phox}$ subunit of NADPH oxidase had been genetically removed (Archer *et al.*, 1999). Rotenone and hypoxia both increase NADH fluorescence and $[Ca^{2+}]_i$, and while selective inhibition of the ETC at either complex I or III does not induce vasoconstriction, it does inhibit HPV in rats (Leach *et al.*, 2001; Waypa *et al.*, 2001). A decrease in chemiluminescence, and thus a decrease in ROS production, and an increase in pulmonary arterial pressure are produced by rotenone, antimycin A and hypoxia, while rotenone and antimycin A also inhibit HPV in rat pulmonary arteries (Archer *et al.*, 1993). In this same tissue, cyanide increases ROS levels and does not cause pulmonary vasoconstriction or effect HPV (Archer *et al.*, 1993), indicating that the O_2 sensor in PASMC is located upstream of cytochrome oxidase.

1.3.4 Significance of NO and CO in oxygen chemoreception

Nitric oxide (NO) and CO are both synthesised in mammalian cells and are capable of acting as chemical messengers. The formation of these compounds is controlled by the intracellular redox state due to the requirement of O_2 for their formation (Moncada *et al.*, 1991; Snyder, 1992). Unlike biogenic amines, CO and NO are not stored in vesicles but are capable of diffusing across membranes with a half-life of a few seconds and, more importantly, have similar characteristics to oxygen in that they bind to heme, usually with a higher affinity than O_2 (Abu-Soud *et al.*, 1996; Migita *et al.*, 1998). Most of the biological actions of NO and CO involve the activation of heme-containing proteins. The following section will discuss the significance that NO and CO may have in oxygen chemoreception.

1.3.4.1 Nitric oxide

NO is produced when L-arginine is converted to L-citrulline by the enzyme nitric oxide synthase (NOS), a heme containing enzyme requiring molecular O_2 for its activation. Three isoforms of NOS exist; neuronal, endothelial and inducible, with the sensory innervation of the carotid body containing the neuronal isoform (Prabhakar *et al.*, 1993; Wang *et al.*, 1993) and the carotid body blood vessels containing the endothelial isoform (Wang *et al.*, 1993). It is unknown whether the glomus cells, or some other component of the carotid body, are responsible for this synthesis of NOS. Neuronal NOS is also localised in nerve fibres innervating chromaffin and ganglion cells, but not in chromaffin cells, of the bovine adrenal medulla (Marley *et al.*, 1995). Hypoxia inhibits NOS activity in the carotid body once P_{O_2} drops below 60 mmHg (Prabhakar *et al.*, 1993) and upregulates endothelial, but not neuronal, NOS gene expression by three-fold after 12 hours of hypoxia

and neuronal, but not endothelial, NOS by nine-fold after 15 days of hypoxia (Prabhakar, 1999).

Various NOS inhibitors are able to augment carotid body sensory discharge *in vitro* (Wang *et al.*, 1994) and NO donors reduce carotid body activity (Prabhakar *et al.*, 1993; Chugh *et al.*, 1994; Wang *et al.*, 1994). These studies suggest that NO is produced under basal conditions and exerts an inhibitory effect on the carotid body. This is confirmed in mice genetically deficient in neuronal NOS which have a more pronounced respiratory response to hypoxia, hyperoxia and cyanide, indicative of augmented chemoreceptor sensitivity (Kline *et al.*, 1998). These mutant mice also display short-term potentiation of breathing after brief episodes of hypoxia and long-term facilitation of breathing following three consecutive hypoxic challenges, implicating endogenous NO as having suppressive effects on ventilation (Kline *et al.*, 2002). NO donors inhibit L-type Ca^{2+} currents, but not K^+ current, independently of voltage in rabbit glomus cells (Summers *et al.*, 1999). No such effects of NO on Ca^{2+} currents are seen in similar experiments in rat carotid bodies, implying species differences in this mechanism (Hatton and Peers, 1996). Nitric oxide donors increase the number of active BK channels in the membrane of guinea-pig proximal colonic myocytes, leading to a small, rapidly oscillating membrane hyperpolarisation (Lang and Watson, 1998). The specific nitrosylation site of these BK channels has been further described as two pairs of cysteine residues surrounded by basic amino acids in the channel α-subunit (Lang *et al.*, 2002).

1.3.4.2 Carbon monoxide

CO is synthesised in mammalian cells by the degradation of heme by the enzyme heme oxygenase (HO), a reaction which requires molecular O_2 to be present and uses

NADPH and cytochrome P-450 reductase as cofactors (Maines, 1997). Two isoforms of HO are most common, an inducible HO-1 and a constitutively expressed HO-2, with HO-2, but not HO-1, expressed in the carotid bodies of cats and rats (Prabhakar *et al.*, 1995).

The inhibitory effect of CO on chemosensory discharge from the carotid body was first noted when the hypoxic suppression of K^+ currents in rabbit glomus cells was reversed by 70% upon the addition of CO to the hypoxic gas mixture (Lopez-Lopez and Gonzalez, 1992). The potent HO inhibitor zinc protoporphyrin IX, but not the structurally similar copper protoporphyrin IX which has negligible effects on HO, increases carotid body sensory activity, an effect reversed by the administration of exogenous CO (Prabhakar *et al.*, 1995). $[Ca^{2+}]_i$ is also increased by zinc protoporphyrin IX, and this effect is reversed by the removal of extracellular Ca^{2+} or administration of exogenous CO (Overholt *et al.*, 1996).

Inhibition of HO activity also causes enhanced respiratory responses to hypoxia and sectioning of the carotid sinus nerve abolishes these effects, indicating that CO directly inhibits the carotid body (Prabhakar, 1999). HO-2 has been localised to endothelial cells and adventitial nerves of blood vessels similarly to NOS (Moncada *et al.*, 1991), and may, like NO, have an endothelial-derived relaxing activity (Zakhary *et al.*, 1996). It is possible, therefore, that CO may also act as a vasodilator, enhancing the oxygenation of the carotid body to further reduce transmitter release. Whether this vasodilation actually has any significant effect on carotid body activity during episodes of hypoxia, when O_2, and therefore CO, levels are reduced, remains to be seen.

It appears therefore, that NO and CO play a role in O_2 sensing, with evidence in the carotid body, AMCCs and vasculature supporting this. NOS and HO perform over a physiologically relevant range of O_2 levels and the effects of NO and CO during normoxia appear inhibitory, keeping the chemosensory activity to a minimum. As O_2 levels drop, the

activities of NOS and HO also decrease, and the increased sensory discharge during hypoxia may in part be due the reduced inhibitory actions of NO and CO. While a substantial body of work has been carried out on this topic no evidence exists yet which directly illustrates whether NO and CO production is actually decreased by hypoxia in O_2 sensing tissues.

1.4 Regulation of catecholamine secretion

The non-neurogenic secretion of catecholamines from the adrenal medulla in direct response to hypoxia was first discovered over forty years ago (Comline and Silver, 1961). This study found that from as early as eight days gestation in sheep, fetal asphyxia induced by umbilical cord occlusion evokes significant increases in the adrenal venous blood catecholamine levels. Prior to the development of adrenal innervation, this catecholamine release is not inhibited by the nicotinic receptor antagonist, hexamethonium, or by spinal chord destruction (Comline and Silver, 1961). Once adrenal innervation does develop, these investigators found that the non-neurogenic response to asphyxia diminishes. As this level of fetal asphyxia was not representative of physiological conditions, similar experiments were repeated using more moderate levels of hypoxaemia with a 60% reduction in fetal arterial P_{O_2} being induced in chronically catheterised fetal sheep *in utero* (Cheung, 1990). The increase in plasma catecholamine levels in 110-120 day old and 130-140 day old fetuses was partially and totally abolished in the presence of hexamethonium in these two respective age groups (Cheung, 1990) confirming the results of Comline and Silver (1961). This more recent study also revealed that the onset of adrenal innervation occurs around 125 days gestation in sheep, as the non-neurogenic hypoxic response does not exist after 130 days gestation.

Non-neurogenic adrenal catecholamine secretion in response to hypoxia also occurs in the rat and calf neonate, where adrenal innervation does not develop until after birth (Comline and Silver, 1966; Seidler and Slotkin, 1985; Seidler and Slotkin, 1986b; Slotkin and Seidler, 1988). As in the fetal sheep, the development of adrenal innervation in these species causes the loss of the non-neurogenic response of this tissue to hypoxia (Comline and Silver, 1966; Slotkin and Seidler, 1988). It appears that some factor released from the splanchnic nerves must be responsible for this suppression of the non-neurogenic hypoxic response, as accelerated development of adrenal innervation by thyroid hormone administration causes this direct response to be suppressed at an earlier stage and severing of the splanchnic nerve causes the non-neurogenic response to remain in rat neonates (Seidler and Slotkin, 1986b; Slotkin and Seidler, 1988). Also, perfused adrenal glands obtained from fetal sheep after adrenal innervation has developed respond to hypoxia *in vitro* (Adams *et al.*, 1996) as do AMCCs isolated from fetal (Rychkov *et al.*, 1998) and adult sheep (Cheung, 1989).

The following section addresses the different factors which can induce and modulate catecholamine secretion from the adrenal medulla. It also focuses on which of these factors might be responsible for the suppression of the non-neurogenic hypoxia-evoked secretion of catecholamines which occurs upon the development of adrenal innervation.

1.4.1 Cholinergic stimulation-induced secretion

Catecholamine secretion from the adrenal medulla is evoked in the mature animal via stimulation of the preganglionic, cholinergic splanchnic nerves which innervate AMCCs. A number of species such as sheep (Comline and Silver, 1961), rat (Santana *et*

al., 1999), cat (Montiel *et al.*, 1995) and dog (Fujimoto *et al.*, 1987) exhibit increased catecholamine secretion as a result of splanchnic nerve stimulation, and physiological stressors such as hypoxia (Seidler and Slotkin, 1985; Seidler and Slotkin, 1986b) and hypoglycaemia (Khalil *et al.*, 1986; Seidler and Slotkin, 1986b) are capable of causing secretion from the adrenal medulla via neural pathways. In both cats and dogs, catecholamine secretion can be induced by chemoreceptor stimulation through perfusion of the carotid body with hypoxic blood (Critchley *et al.*, 1980). Sensory afferent fibres of the carotid body project to the brainstem through the carotid sinus nerve (Donoghue *et al.*, 1984) and these areas of the hypothalamus and brainstem control the splanchnic nerve output to the adrenal medulla (Robinson *et al.*, 1983; Goadsby, 1985).

Nicotinic and muscarinic receptors in the AMCC plasma membrane are activated by the neurotransmitter, acetylcholine (ACh), which is released from splanchnic nerve terminals upon sympathetic stimulation, triggering catecholamine secretion. There is a large degree of species variation in the number of nicotinic and muscarinic receptors present in AMCC plasma membranes. The secretion of catecholamines from the bovine (Livett *et al.*, 1983) and sheep (Butler *et al.*, 1995) adrenal is controlled predominantly by nicotinic stimulation, while secretion from rat (Wakade and Wakade, 1983) and dog (Critchley *et al.*, 1986) adrenal glands is activated by stimulation of both muscarinic and nicotinic receptors.

1.4.1.1 Secretion associated with nicotinic stimulation

Secretion caused by nicotinic receptor stimulation is dependent upon extracellular Ca^{2+} (Mizobe and Livett, 1983) and Na^{+} entering the cell through the nAChRs (Vernino *et al.*, 1994; Khiroug *et al.*, 1997), causing a large membrane depolarisation and the opening

of VGCCs (Aunis and Langley, 1999). Upon sustained activation of nAChRs, desensitisation of the receptor can occur in which a given stimulus no longer has the same effect it had during previous stimulations. This desensitisation is due to the effect increased $[Ca^{2+}]_i$ has on stabilizing the conformation of nAChRs and retarding their recovery to full activity (Khiroug *et al.*, 1997). This influx of Ca^{2+} ions is initially spatially localised to the subplasmalemmal area of the cell before spreading throughout the cell during a sustained stimulation (Cheek *et al.*, 1989). The main Ca^{2+} channel subtype associated with the nicotinic control and modulation of catecholamine secretion from bovine and cat AMCCs is the L-type channel, as dihydropyridines have been shown to greatly reduce secretion induced by nicotinic stimulation of AMCCs (Lopez *et al.*, 1989; Owen *et al.*, 1989).

The increased level of nAChR stimulation in the adrenal medulla by acetylcholine, which occurs upon the development of adrenal innervation, would be a likely reason for the suppression of the non-neurogenic response to hypoxia. This is not the case, however, as inhibition of nAChR activity with hexamethonium does not fully suppress the non-neurogenic hypoxic response in 110-120 day old sheep fetuses, when adrenal innervation is only partially developed (Cheung, 1990), indicating that some factor other than acetylcholine is responsible for this suppression.

1.4.1.2 Secretion associated with muscarinic stimulation

Muscarinic stimulation of AMCCs has two main effects which can induce adrenal catecholamine secretion, mobilisation of Ca^{2+} from internal stores and membrane depolarisation. Unlike nAChR stimulation, catecholamine secretion induced by muscarinic acetylcholine receptor (mAChR) stimulation in AMCCs does not require extracellular Ca^{2+} in order to function in the rat (Neely and Lingle, 1992a; Prakriya *et al.*, 1996) and guinea

pig (Misbahuddin and Oka, 1988). In single rat AMCCs, muscarine produces a transient increase in $[Ca^{2+}]_i$, reaching peak levels of 3-5 μM (Prakriya *et al.*, 1996), which is not blocked by either Co^{2+} or removal of extracellular Ca^{2+}, indicating the source of this Ca^{2+} is from intracellular stores (Kao and Schneider, 1985; Neely and Lingle, 1992a). Muscarine-induced elevation of $[Ca^{2+}]_i$ is associated with polyphosphoinositide breakdown and increased inositol trisphosphate (IP_3) levels in AMCCs (Cheek and Burgoyne, 1985; Malhotra *et al.*, 1988), prompting the release of Ca^{2+} from IP_3-sensitive intracellular Ca^{2+} stores (Stoehr *et al.*, 1986). This release of intracellular Ca^{2+} causes the opening of $K_{(Ca)}$ channels in single rat and guinea pig AMCCs (Inoue and Kuriyama, 1990; Neely and Lingle, 1992a; Inoue *et al.*, 1995; Barbara *et al.*, 1998) and rat adrenal slices (Inoue and Kuriyama, 1990; Neely and Lingle, 1992a; Inoue *et al.*, 1995; Barbara *et al.*, 1998) which results in a brief membrane hyperpolarisation (Inoue and Kuriyama, 1990; Inoue *et al.*, 1995).

The application of muscarine also suppresses the 'M' current (I_M), a subthreshold voltage- and time-dependent K^+ current first described in sympathetic neurons (Brown and Adams, 1980). As the channels underlying I_M are active near the resting membrane potential, inhibition of these channels results in a small membrane depolarisation and decrease in membrane conductance, with both of these effects leading to increased cell excitability. Recently, subtypes of the KCNQ K^+ channel family were identified as those responsible for this I_M in both a heterologous and neuronal preparation (Wang *et al.*, 1998), with phosphatidylinositol 4,5-bisphosphate (PIP_2) synthesis required for recovery from this muscarine-induced channel inhibition (Suh and Hille, 2002). This depolarisation of the cell membrane upon mAChR stimulation has been observed in guinea pig, rat and pig AMCCs (Inoue and Kuriyama, 1990; Inoue and Kuriyama, 1991; Forsberg *et al.*, 1995; Barbara *et al.*, 1998).

Several factors argue against any role for muscarinic stimulation in suppressing the direct hypoxic response. Firstly, muscarinic stimulation causes a membrane depolarisation and increases the input resistance of AMCCs, effects which would make these cells more excitable, not less. Secondly, the suppression of the non-neurogenic hypoxic response has been well documented in the sheep adrenal gland (Comline and Silver, 1961; Cheung, 1990) a tissue which displays no muscarinic influence on secretion, as illustrated by the 99% reduction in acetylcholine-induced catecholamine secretion from the fetal and adult sheep adrenal caused by the application of the nicotinic antagonist hexamethonium (Butler *et al.*, 1995).

1.4.2 Effects of non-cholinergic stimulation on catecholamine secretion

In rat and bovine whole perfused adrenal glands, catecholamine secretion induced by electrical stimulation of the splanchnic nerves is frequency dependent, increasing in a linear fashion from low frequencies (0.5-3.0 Hz) and reaching a plateau at 10 Hz (Wakade, 1981; Marley *et al.*, 1993). When nicotinic and muscarinic input is blocked during high frequency stimulation, 20-25% of the control level of catecholamine secretion remains (Marley *et al.*, 1993), with this residual level of secretion being attributed to a non-cholinergic component of adrenal nerve stimulation. The following section is a brief account of the main non-cholinergic transmitters which can induce secretion from the adrenal medulla, the mechanisms by which they modulate catecholamine secretion and their possible roles in suppressing the non-neurogenic hypoxic response which develops upon adrenal innervation.

1.4.2.1 Opioid peptides

The enkephalin (Enk) containing peptides are located within AMCCs and in splanchnic innervation (Varndell *et al.*, 1982; Wilson *et al.*, 1982; Kobayashi *et al.*, 1985). Stimuli such as splanchnic nerve stimulation, nicotinic stimulation or physiological stress all result in the secretion of not only catecholamines from the adrenal medulla, but also Enk-containing peptides (Livett *et al.*, 1981; Bloom *et al.*, 1988; Kimura *et al.*, 1988). As well as this, several opioid peptide receptor subtypes have been identified in the membranes of AMCCs (Dumont and Lemaire, 1984; Castanas *et al.*, 1985a; Castanas *et al.*, 1985b; Bunn *et al.*, 1988). Opioid peptides inhibit Ca^{2+} uptake in response to nicotinic stimulation in cultured bovine AMCCs (Bunn and Dunkley, 1991), causing a reduction in catecholamine secretion from these cells (Saiani and Guidotti, 1982). These effects are thought to be caused by the inhibitory actions of opioid peptides on Ca^{2+} currents (Twitchell and Rane, 1993; Albillos *et al.*, 1996a) and the augmentation of $K_{(Ca)}$ current, mainly BK current, in bovine AMCCs (Twitchell and Rane, 1994).

The endogenous opioid peptides met-enkephalin, leu-enkephalin, metorphamide, dynorphin and dynorphin B all significantly reduce the level of catecholamine secretion from single bovine AMCCs during nicotinic stimulation (Marley *et al.*, 1986a; Marley *et al.*, 1986b). Both dynorphin and metorphamide are capable of protecting bovine AMCCs against desensitisation of the nicotinic response, but are considerably more potent at inhibiting nicotine-induced secretion (Marley and Livett, 1987). Given the reductions in catecholamine secretion, Ca^{2+} uptake and Ca^{2+} current amplitude and increases in K^+ current amplitude caused by endogenous opioids, and the fact that they are released from splanchnic nerve terminals and co-secreted with catecholamines, it is possible that these peptides are responsible for the suppression of the non-neurogenic hypoxic response upon the development of adrenal innervation.

1.4.2.2 VIP and PACAP

Vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) are regulatory peptides which have N-terminal sequences that share a 68% homology (Miyata *et al.*, 1990) and are widely distributed throughout the body (Nussdorfer and Malendowicz, 1998). The receptors for these two compounds share many biological properties and hence, are named PACAP/VIP receptors (PVRs). PVRs are G protein-coupled receptors which activate adenylate cyclase and phospholipase C (PLC)-dependent signaling pathways (Nussdorfer and Malendowicz, 1998). VIP and PACAP are synthesised by AMCCs and both neuropeptides have binding sites on extrinsic nerve fibres (Nussdorfer and Malendowicz, 1998). In the sheep, adrenal VIP content increases from day 80 to day 110-130 of gestation, with a significant fall occurring at day 140 immediately prior to term (Cheung and Holzwarth, 1986). VIP release by perfused adrenals is sizeably increased upon splanchnic nerve stimulation (Bloom *et al.*, 1988) and splanchnic nerve section lowers VIP immunoreactivity in the rat adrenal medulla by 70% (Hinson and Kapas, 1996). PACAP-positive staining is located in nerve fibres and the noradrenaline-containing cells of the rat adrenal medulla (Frodin *et al.*, 1995; Shiotani *et al.*, 1995).

VIP and PACAP promote catecholamine secretion in AMCCs both *in vivo* and *in vitro* (Cheung and Holzwarth, 1986; Malhotra and Wakade, 1987; Edwards and Jones, 1993; Edwards and Jones, 1994; Watanabe *et al.*, 1995) with PACAP being a considerably more potent stimulant of catecholamine release than VIP (Watanabe *et al.*, 1995). Both these peptides stimulate cAMP formation, phosphoinositide metabolism and Ca^{2+} release from intracellular stores in AMCCs, with only PACAP able to stimulate Ca^{2+} influx through VGCCs (Malhotra *et al.*, 1988; Malhotra *et al.*, 1989; Bunn *et al.*, 1990; O'Farrell and Marley, 1997). In bovine AMCCs, PACAP initiates catecholamine secretion and also increases TH activity threefold within ten minutes exposure through cAMP formation and

PKA activation (Marley *et al.*, 1996; McKenzie and Marley, 2002). The fact that these two peptides both initiate catecholamine secretion makes them unlikely to be responsible for suppressing non-neurogenic hypoxic catecholamine secretion.

1.4.2.3 Substance P

The role of substance P (SP) in the adrenal medulla, unlike VIP and PACAP, is classed as that of a neuromodulator, not a neurotransmitter, as SP does not effect secretion by itself and requires the presence of a nicotinic agonist in order to have an effect on secretion (Livett and Marley, 1993). The two distinct actions of SP on the AMCC nicotinic response are an inhibition of secretion evoked by nicotinic agonists (Livett *et al.*, 1979) and protection against desensitisation of the nicotinic response (Boksa and Livett, 1984). Catecholamine secretion induced by prolonged electrical stimulation is facilitated by SP released from sensory nerve fibres, an effect reversed if adrenal gland SP-containing nerve fibres have been previously destroyed (Zhou and Livett, 1990). Total adrenal SP content correlates with gestational age in the fetal sheep with the adrenal content of SP being highest in the young fetus and decreasing progressively towards term, while in the adult, adrenal levels are significantly lower than those measured in the fetus (Cheung, 1989).

The mechanism of SP modulation of nicotinic stimulation is thought to involve a direct interaction with the nicotinic receptor-ionophore complex. This is because the antagonist actions of SP on secretion are non-competitive with nicotinic agonists such as acetylcholine or DMPP (Livett and Marley, 1993), and SP does not effect catecholamine secretion induced through mechanisms which bypass the nicotinic receptor ionophore complex such as muscarine, veratridine, high external K^+ concentrations or Ca^{2+} ionophores (Boksa and Livett, 1984; Zhou *et al.*, 1991). Given that SP levels in the adrenal

gland decrease as the fetus nears term and that the action of this peptide is directly through the nicotinic receptor, it appears unlikely to be responsible for the alleviation of the direct hypoxic response, as this response only occurs prior to adrenal innervation and does not involve nicotinic receptor activation.

1.4.2.4 Angiotensin II

Angiotensin II (Ang II) has been shown to initiate the release of adrenal catecholamines (Bunn and Marley, 1989; Livett and Marley, 1993) in relatively small amounts in single cells and whole perfused adrenal glands (Bunn and Marley, 1989; Powis and O'Brien, 1991). This stimulation of exocytosis in the adrenal medulla by Ang II is greater in noradrenaline-secreting cells than in adrenaline-secreting cells (Choi *et al.*, 1993). Using radioligand binding and autoradiography, Ang II receptors have been identified in the adrenal medulla and isolated AMCCs (Quirion *et al.*, 1983; Healy *et al.*, 1985; Marley *et al.*, 1989). Catecholamine secretion evoked by splanchnic nerve stimulation in anaesthetized dogs is reduced significantly in the presence of the Ang II receptor antagonist, saralasin (Foucart *et al.*, 1991), suggesting that Ang II is released from splanchnic nerve terminals.

In AMCCs, Ang II potentiates exocytosis by increasing the influx of extracellular Ca^{2+}, activating PLC and mobilising Ca^{2+} from internal stores, with PKC inhibitors decreasing these facilitatory effects of Ang II (Bunn *et al.*, 1990; Teschemacher and Seward, 2000). It is the relatively small proportion of increased $[Ca^{2+}]_i$ from extracellular, rather than intracellular, sources which is important in stimulating Ang II-evoked catecholamine secretion (Bunn and Marley, 1989; McMillian *et al.*, 1992) and this appears

to occur through N-type rather than L-type Ca^{2+} channels in bovine AMCCs (McMillian *et al.*, 1992).

Ang II fulfils some of the requirements for being the peptide responsible for the alleviation of the direct hypoxic response, as it is actively released from the splanchnic nerve terminals and effects the level of catecholamines secreted from the adrenal medulla. However, this effect of Ang II on secretion is stimulatory, rather than inhibitory, making it unlikely to cause the suppression of non-neurogenic hypoxic catecholamine secretion as adrenal innervation develops.

1.4.2.5 Histamine

Histamine is a powerful G protein-coupled secretagogue in AMCCs, comparable in potency to nicotinic agonists and PACAP (Donald *et al.*, 2002). Histamine acts on AMCCs similarly to PACAP in that they both induce increased TH activity, stimulate PLC, increase IP_3 formation, mobilise Ca^{2+} from intracellular stores and evoke catecholamine secretion (Bunn *et al.*, 1995; McKenzie and Marley, 2002) and rely on extracellular Ca^{2+} entry through VGCCs to produce a sustained secretory response (O'Farrell and Marley, 1999). AMCCs contain the H1 histamine receptor, and histamine-induced IP_3 accumulation and catecholamine secretion is greater in adrenaline-, rather than noradrenaline-containing AMCCs, likely due to the three times higher density of H1 receptors in adrenaline-containing cells (Choi *et al.*, 1993). The dose-dependent secretion of catecholamines produced by histamine is not affected by hexamethonium, indicating its actions are not through the nicotinic receptor ionophore complex (Livett and Marley, 1986; Kitamura *et al.*, 1996). Histamine causes a transient membrane hyperpolarisation due to intracellular Ca^{2+} release opening SK channels, and this is followed by the closure of K^+ channels

causing a sustained depolarisation of around 7 mV and increased discharge of APs in bovine AMCCs (Wallace *et al.*, 2002). The rates of catecholamine secretion and TH phosphorylation produced by histamine are enhanced when SK channels are blocked by apamin (Kitamura *et al.*, 1996).

SK channel closure during hypoxia causes membrane depolarisation in rat AMCCs (Lee *et al.*, 2000), and this could be offset by the histamine-induced hyperpolarisation caused by the opening of SK channels. This activation of SK channels is short-lived, however, and followed by a membrane depolarisation caused by the closure of K^+ channels (Wallace *et al.*, 2002). Catecholamine secretion evoked by splanchnic nerves stimulation is not modified by a combination of H1 and H2 receptor antagonists (Borges, 1994), indicating that the histamine that elicits catecholamine release from rat adrenals comes from blood circulation, not from splanchnic nerves. These factors suggest that histamine is unlikely to be the factor released from splanchnic nerves which suppresses the non-neurogenic response to hypoxia.

This concludes the review of the literature pertaining to the topic of hypoxia-evoked secretion of catecholamines in the fetal and postnatal adrenal medulla. The following sections will focus on the experimental component of this thesis and is categorised into four separate sections, each concentrating on a specific topic. These topics are the identification of the oxygen-sensitive ion channels in AMCCs, the intracellular mechanisms responsible for the hypoxic response in AMCCs, the involvement of opioid peptides in suppressing the direct hypoxic response, and possible developmental changes in the levels of different Ca^{2+} channels in fetal and adult AMCCs and the effect of these changes on Ca^{2+} entry and $K_{(Ca)}$ channel activation.

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

2.1 Introduction

In the adrenal medulla of the sheep fetus and newborn rat, prior to the development of functional innervation, hypoxia acts directly on the AMCCs to stimulate catecholamine secretion (Comline and Silver, 1961; Slotkin and Seidler, 1988; Cheung, 1990). This direct response of the adrenal medullary cells to hypoxia results in a re-direction of blood to the heart, brain and adrenal glands, and is crucial for the survival of the fetus (Phillippe, 1983). Once innervation has developed (by 130 days gestation in the sheep) the direct response of the adrenal medullary cells to low P_{O_2} disappears (Cheung, 1990). It is restored, however, upon denervation of the adrenal gland, as demonstrated by the hypoxia-evoked secretion of catecholamines from the perfused whole adrenal gland of the adult sheep (Adams *et al.*, 1996).

It is believed that the mechanism of O_2 sensing in the AMCCs is similar to that in the glomus cells of the carotid body, the major chemoreceptor cells in the adult mammals, where it involves K_{O_2} channels. These K_{O_2} channels close under hypoxic conditions, leading to membrane depolarisation, opening of VGCCs and exocytosis of neurotransmitters subsequent to the elevation of the cytosolic $[Ca^{2+}]_i$ (reviewed by Prabhakar, 2000). In both the carotid body and adrenal medulla, there is debate over what types of channels are responsible for the initial hypoxia-evoked depolarisation of the cell membrane. This is due mainly to the high degree of species variability associated with the identity of known K_{O_2} channels.

In AMCCs of the sheep, a current produced by $K_{(Ca)}$ channels is reduced during hypoxia (Rychkov *et al.*, 1998), but the types of $K_{(Ca)}$ channels involved have not been fully identified. BK channels are present in the AMCCs of many species (Marty and Neher, 1985; Neely and Lingle, 1992b) and at least part of the O_2-sensitive current in the sheep

adrenal medulla is through BK channels (Rychkov *et al.*, 1998). Closure of these channels seems unlikely to cause the hypoxia-evoked depolarisation, however, as BK channels are strongly voltage dependent and are usually closed at resting potential (Lippiat *et al.*, 2000). $K_{(Ca)}$ channels which are open at resting potential and may be responsible for the sensitivity of sheep AMCCs to hypoxia are intermediate conductance K^+ (IK) channels and SK channels. IK channels are not present in the human adrenal medulla (Ishi *et al.*, 1997) and do not appear to have been described in the adrenal of other species. Voltage-independent SK channels have been found in bovine and rat AMCCs (Marty and Neher, 1985; Neely and Lingle, 1992b), and mRNA for SK2 channels, a subtype of the SK channel family, has been isolated from the rat adrenal medulla (Kohler *et al.*, 1996). SK channels were recently shown to play the role of the K_{O_2} channel in rat AMCCs (Lee *et al.*, 2000).

In the following set of experiments, the results of patch clamp studies and intracellular Ca^{2+} measurements show that more than one type of $K_{(Ca)}$ channel is involved in the response of the sheep adrenal medulla to hypoxic stimuli, with the SK channel responsible for the initial depolarisation. The manuscript relating directly to the experiments described in this chapter, which was published in *The American Journal of Physiology,* is attached for further reference as Appendix 2 at the rear of this thesis.

2.2 Materials and Methods

2.2.1 Isolation of adrenal chromaffin cells

2.2.1.1 Fetal cells

Merino cross ewes between 137 and 142 days gestation (term is 147 ± 3 days) were used for these experiments which were approved by the Adelaide University Animal Ethics Committee. Ewes were killed with intravenous pentobarbitone (8.1 g), and the fetus was removed through a laparotomy incision and decapitated. The fetal adrenal gland was removed and the medulla, dissected free of the cortex, was minced and incubated at 37°C for 45 min in a Ca^{2+}-free Locke's solution consisting of (mM): NaCl, 154; KCl, 5.6; $NaHCO_3$, 3.6; glucose, 5.6; HEPES, 5.0; pH 7.4, supplemented with collagenase (Worthington Type II, 0.1%) and deoxyribonuclease (Sigma, Type IV; 100 U ml^{-1}). Repeated pipetting mechanically dispersed the tissue and the cells were washed twice in Ca^{2+}-free Locke's solution. The cells were then resuspended in DMEM containing 10% charcoal-absorbed fetal bovine serum (Trace, Australia), 0.1 μM dexamethasone, 100 U ml^{-1} penicillin and 0.5 mg ml^{-1} streptomycin, plated on glass coverslips and maintained in culture.

2.2.1.2 Adult cells

Adult AMCCs were isolated by the same technique as that for fetal cells obtained, using either adrenal glands from the pregnant ewe or glands from a local abattoir collected from freshly killed merino sheep, placed in Ca^{2+}-free Locke's solution and kept on ice for a

maximum of one hour until gland dissection was carried out. The only modification in the cell isolation protocol was that the collagenase concentration was doubled to 0.2%.

2.2.2 Electrophysiology

Whole-cell recordings were conducted on cells 1 to 3 days after plating, always at room temperature. Pipettes with a resistance between 2 and 4 MΩ were used and series resistance was 80-95% compensated. Currents were recorded with an EPC-9 amplifier (HEKA), data acquisition and analysis was performed on an IBM compatible computer using Pulse and Pulsefit software (version 8.11, HEKA). Corrections for liquid junction potential between the bath and electrode solutions were estimated by JPCalc (Barry, 1994). AMCCs, in a bath of approximately 500µl, were superfused continuously at 2-3 ml min^{-1} with a solution containing (mM): NaCl, 140; KCl, 4; $CaCl_2$, 2; $MgCl_2$, 2; HEPES, 10; with pH adjusted to 7.4 with NaOH. The bath solution was equilibrated with either room air or 100% N_2 flowing through rubber jacketed tubing. P_{O_2} was measured with an O_2 microelectrode (World Precision Instruments, USA) placed near the recording site. With hypoxic superfusion, P_{O_2} reached a level of 7-10 mmHg. For electrophysiological recordings cells were exposed to these low P_{O_2} levels for between 1-5 minutes. When Ca^{2+} currents were recorded, cells were bathed in a solution containing (mM): $CaCl_2$, 10; $MgCl_2$, 2; NaCl, 70; TEA-Cl, 70; HEPES, 10; TTX, 3 x 10^{-4}, 5-Nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), 0.1. Whole cell K^+ currents were recorded using a pipette filling solution containing (mM): KCl, 60; K-glutamate, 75; $MgCl_2$, 2; Na_2ATP, 2; EGTA, 2; HEPES, 10; adjusted to pH 7.2 with KOH. For perforated patch experiments, Na_2ATP and EGTA were omitted from this pipette solution and amphotericin (500 µg/ml) was added. When recording Ca^{2+} currents the pipette filling solution consisted of (mM):

TEA-Cl, 60; Cs-glutamate, 65; MgATP, 4; EGTA, 10; HEPES, 10; adjusted to pH 7.2 with TEA-OH.

2.2.3 Calcium Imaging

AMCCs were loaded with the Ca^{2+} indicator by incubating coverslips in a mixture of 20 μM Fluo-3 AM and 0.0025% Pluronic acid in DMEM at 37°C for 30 minutes. Confocal microscopy with a water immersion lens was applied to single cells using an argon ion laser (Optiscan, Australia) scanning at a peak of 488 nm. Images were captured using confocal software (F900e, version 1.6, Optiscan, Australia) and analysed using Scion Image (Scion Corporation, USA). Laser intensity was reduced to 1% of maximum by use of neutral density filters and the number of scans per cell kept to a minimum to avoid photobleaching. Changes in intracellular Ca^{2+} levels were taken as the ratio of the increase in the mean pixel value of the whole cell compared to that in the control period, where the pixel values are a grey scale ranging from zero to 255. All values of increased fluorescence were taken 60 seconds after the start of application of either hypoxic solution or specific channel antagonists through the perfusion system. This ratio is calculated according to the equation:

$$\mathbf{R = (F-F_{min}) / F_{min}}$$

where: R = ratio of Ca^{2+} fluorescence change

F_{min} = mean fluorescence intensity level during control period

F = mean fluorescence intensity level following 1 min of stimulation

This ratio was then multiplied by 100 to express any fluorescence changes as a percentage value of the resting fluorescence level.

2.2.4 Fluorescence Immunohistochemistry

2.2.4.1 Tissue preparation

Whole adult adrenal glands were removed from adult ewes as previously described in Section 2.2.1.2. Upon removal, the glands were washed in Ca^{2+}-free Locke's solution, end sections severed and the gland placed in a modified Zamboni fixative solution (8% paraformaldehyde, 15% saturated picric acid, 0.1M phosphate buffer; pH 7.0) overnight at 4°C. After fixation, excess picric acid was cleared with four 10 min washes in dimethyl sulfoxide followed by three 10 min washes in 0.1M phosphate buffered saline (PBS). Glands were then immersed in PBS containing 0.1% sodium azide and 30% sucrose overnight at 4°C. Glands were then placed in a cryomold tissue holder, covered in optimal cutting temperature compound (OCT; Tissue Tek, Miles Inc. Elkhart, IN), frozen using pre-cooled isopentane and stored at -20°C for later use.

2.2.4.2 Immunohistochemistry

Adrenal gland segments were sectioned (15 μM) using a cryostat (Lieca CM 1800, Nussloch, Germany) at -20°C, placed on electrostatic adhesive slides (two sections per slide) and stored at -20°C. For staining, sections were washed in PBS and then incubated in 10% normal donkey serum (NDS; Jackson ImmunoResearch, Australia) at room temperature for 30 minutes to prevent non-specific binding of antibodies. All antibodies were diluted in antibody diluent consisting of 290mM NaCl, 7.5mM Na_2HPO_4, 2.5mM $NaH_2PO_4.2H_2O$, 0.001% sodium azide; pH 7.1. Sections were incubated overnight in a humidified chamber at 4°C with either SK2 or SK3 (Alomone Laboratories, Jerusalem, Israel, 1:2000 dilution) primary antibodies (raised in rabbit), TH (DiaSorin Inc., USA, diluted 1:1000) primary antibody (raised in mouse) and 10% NDS. These SK channel

antibodies were raised against amino acid residues 542-559 and 2-21 of human SK2 and SK3, respectively, and these sequences are not present in other SK channel isoforms (Alomone). These sequences are highly homologous with other species such as human (89% homology), mouse (95%) and chicken (78%) for SK2 and mouse (100%) and rat (100%) for SK3 (Alomone). The specificity of the antibodies was verified by competitively blocking the immunofluorescence signal with an SK2- or SK3-specific antigen peptide (Alomone) used as recommended by the manufacturers.

After incubation with primary antibodies, sections were washed three times for 10 min in PBS, and incubated with species specific secondary antibodies for 40 min at 37°C. The rabbit anti-SK was labeled with a biotinylated donkey anti-rabbit secondary antibody (diluted 1:50, Jackson ImmunoResearch, Australia) and the mouse anti-TH was labeled with donkey anti-mouse coupled to the fluorphore fluorescein isothiocyanate (FITC; diluted 1:20, Jackson ImmunoResearch, Australia). Sections were washed once in PBS before being incubated in tetramethylrhodamine isothiocyanate (TRITC)-streptavidin (diluted 1:100, Jackson ImmunoResearch, Australia) at 37°C for 40 minutes. Prior to viewing, slides were washed one final time with PBS and sealed with a coverslip using a mounting medium containing 1% p-phenylenediamine and 80% glycerol in 0.1M PBS; pH 8.0.

2.2.4.3 Cell imaging techniques

Prepared slides were viewed under an Olympus BX50 fluorescent microscope fitted with selective filters for discriminating between different labels (FITC; U-NIBA (Olympus) 470-490nm and TRITC; U-NG (Olympus) 530-550nm). Images were digitally captured using a SPOT RT CCD camera (Diagnostic Instruments Inc., USA) connected to

an IBM computer and analysed using SPOT RT imaging software package (Diagnostic Instruments Inc., USA). The micrographs captured allowed visualisation of SK and TH immunoreactive cells which fluoresced green and red, respectively. Captured images were processed for brightness and contrast using Adobe Photoshop and prepared for publication using Adobe Pagemaker 6.5.

2.2.5 Drugs

Apamin extracted from bee venom, d-tubocurarine, TEA-chloride, NPPB, amphotericin and DMEM were obtained from Sigma Chemicals, USA. Fluo-3 AM and Pluronic F-127 were supplied by Molecular Probes, USA.

2.2.6 Statistics

Results are expressed as means ± SEM. Two group comparisons, such as the effect of hypoxia, were tested by Student's paired t-tests. The significance of differences between treatments was tested by an ANOVA and Tukey's *post hoc* test. $p<0.05$ was taken as the minimum level of significance.

2.3 Results

2.3.1 Oxygen-sensitive K^+ currents in fetal adrenal chromaffin cells

Of the 40 AMCCs tested from 137-142 day fetal sheep, 32 displayed outward currents which were suppressed significantly by acute hypoxia. The I-V relationship in these cells showed a local maximum at about +40 mV, a characteristic of $K_{(Ca)}$ currents, and hypoxic suppression was significant in the region of 0 to 60 mV ($p<0.05$), the region where the Ca^{2+}-dependent current is obvious (Fig. 2.1A). The peak current in these cells was reduced by 36 ± 7% by hypoxia at 40 mV (n=11). This hypoxic suppression of current was reversible upon return to normoxic conditions and these cells were classed as O_2-sensitive. In perforated patch recording mode, similar currents were present and hypoxia produced a similar reduction in the outward current which was reversed on return to normoxic conditions (results not shown).

2.3.2 Effect of hypoxia on voltage-dependent Ca^{2+} current

The reduction in the $K_{(Ca)}$ current by hypoxia could be through a direct action on the K^+ channels or secondary to a reduced Ca^{2+} influx. The I-V relationship of Ca^{2+} current in fetal AMCCs was not shifted by hypoxia and at none of the voltage steps was a significant difference seen between the Ca^{2+} current amplitude in control and hypoxic conditions. The peak current amplitude of Ca^{2+} current observed when stepping from a holding potential of -80 mV to 10 mV in control conditions was -395 ± 36 pA (Fig. 2.1B, n=7) which was not significantly different to the current amplitude at 10 mV of -391 ± 36 pA seen in hypoxia (n=7).

2.3.3 Contribution of different types of K^+ channels to the oxygen-sensitive current.

Apamin, an inhibitor of SK channels, reduced the outward current in fetal AMCCs (Fig. 2.2A). This reduction was significant at voltages from 0 to 60 mV, and at 40 mV, 200 nM apamin reduced the current by 36 ± 8% (n= 9). Comparison of the effects of different concentrations of apamin on the K^+ current showed that 1 nM, 10 nM and 200 nM all caused significant decreases in whole cell current from 20 to 60 mV ($p<0.05$) but at no potential were the effects of these three concentrations different from each other (Fig. 2.2D).

In the presence of apamin, the absolute reduction of K^+ currents by hypoxia was much smaller, indicating that SK channels in the AMCCs of fetal sheep are sensitive to oxygen concentration. Application of 200 nM apamin did not eliminate K_{O_2} currents in these cells completely; an O_2-sensitive component persisted at voltages ranging from 0 to 50 mV (paired t-test; $p<0.05$; Fig. 2.2A). The combination of apamin and hypoxia resulted in a current reduction to 40 ± 7% of the control at 40 mV. This level of current suppression was less than the sum of that caused by hypoxia (36%) or apamin (36%) separately, indicating an overlap in the channels which are blocked by apamin and hypoxia. The outward current remaining in the presence of apamin was almost completely blocked by application of 1 mM TEA in the external solution. Dependence on external Ca^{2+} (not shown) and complete block by relatively low concentrations of TEA was consistent with this current being mediated by BK channels. In the presence of both apamin (200 nM) and TEA (1 mM), no O_2-sensitive current remained (Fig. 2.2C). These results indicate that the AMCCs of fetal sheep possess at least two types of $K_{(Ca)}$ channels: SK and BK channels, and that they both are O_2-sensitive.

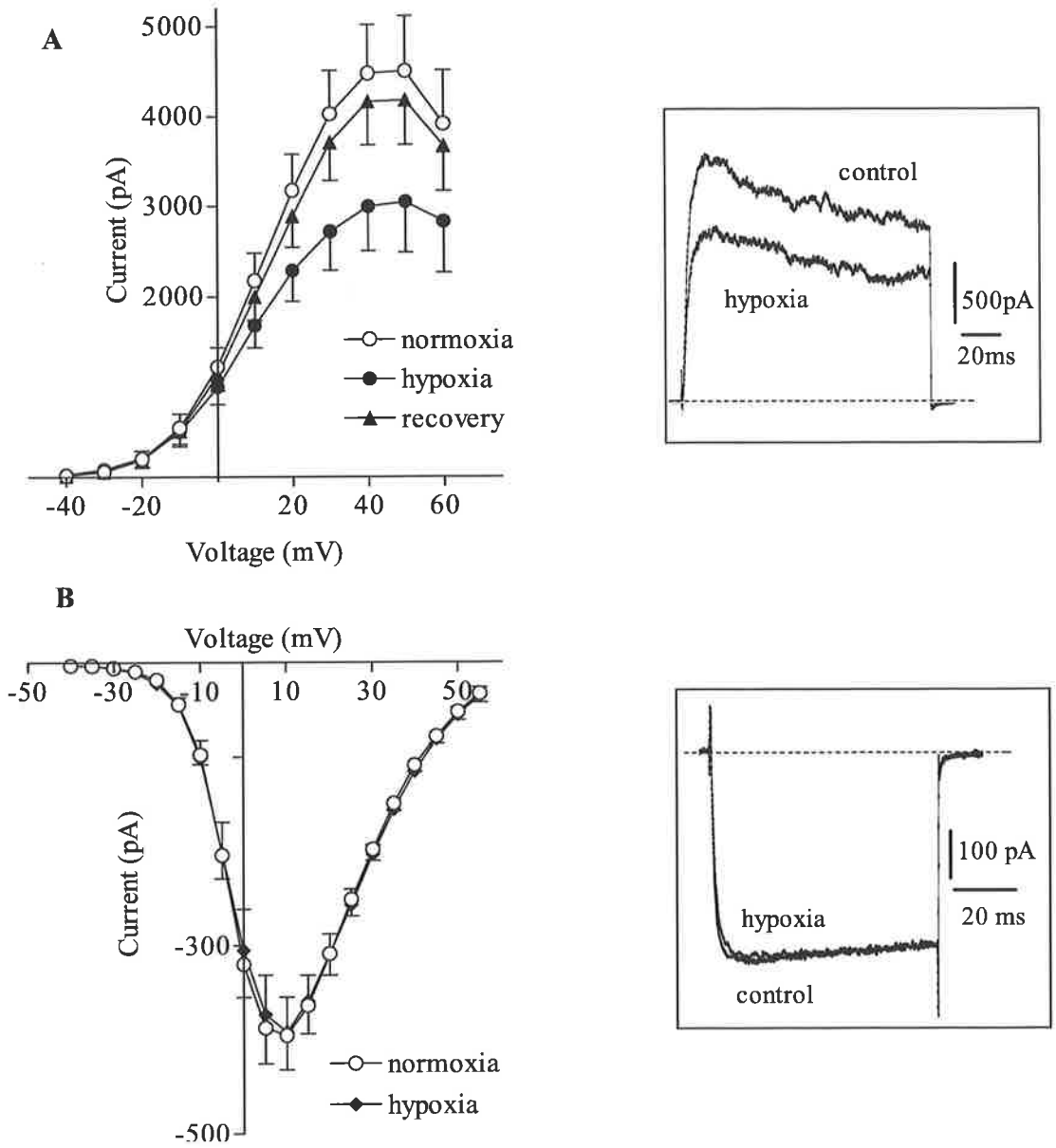


Figure 2.1: Effect of hypoxia on currents in fetal sheep AMCCs. **A** I-V relation of K^+ currents in control conditions (○), during hypoxia (●), and upon return to normoxia (▲) (n=11). Cells held at -80 mV and stepped to potentials from -40 to +60 mV for 200 ms. *Inset* Example trace of a whole cell current evoked by a single step pulse from –80 mV to +40mV in normoxic and hypoxic conditions. **B** The I-V relationship of Ca^{2+} current during normoxia (○) and hypoxia (◆) (n=7). Cells were held at -80 mV and stepped to potentials from -40 to +55 mV for 100 ms. *Inset* Example trace of a Ca^{2+} current evoked by a single step pulse from -80 mV to +10mV in normoxic and hypoxic conditions.

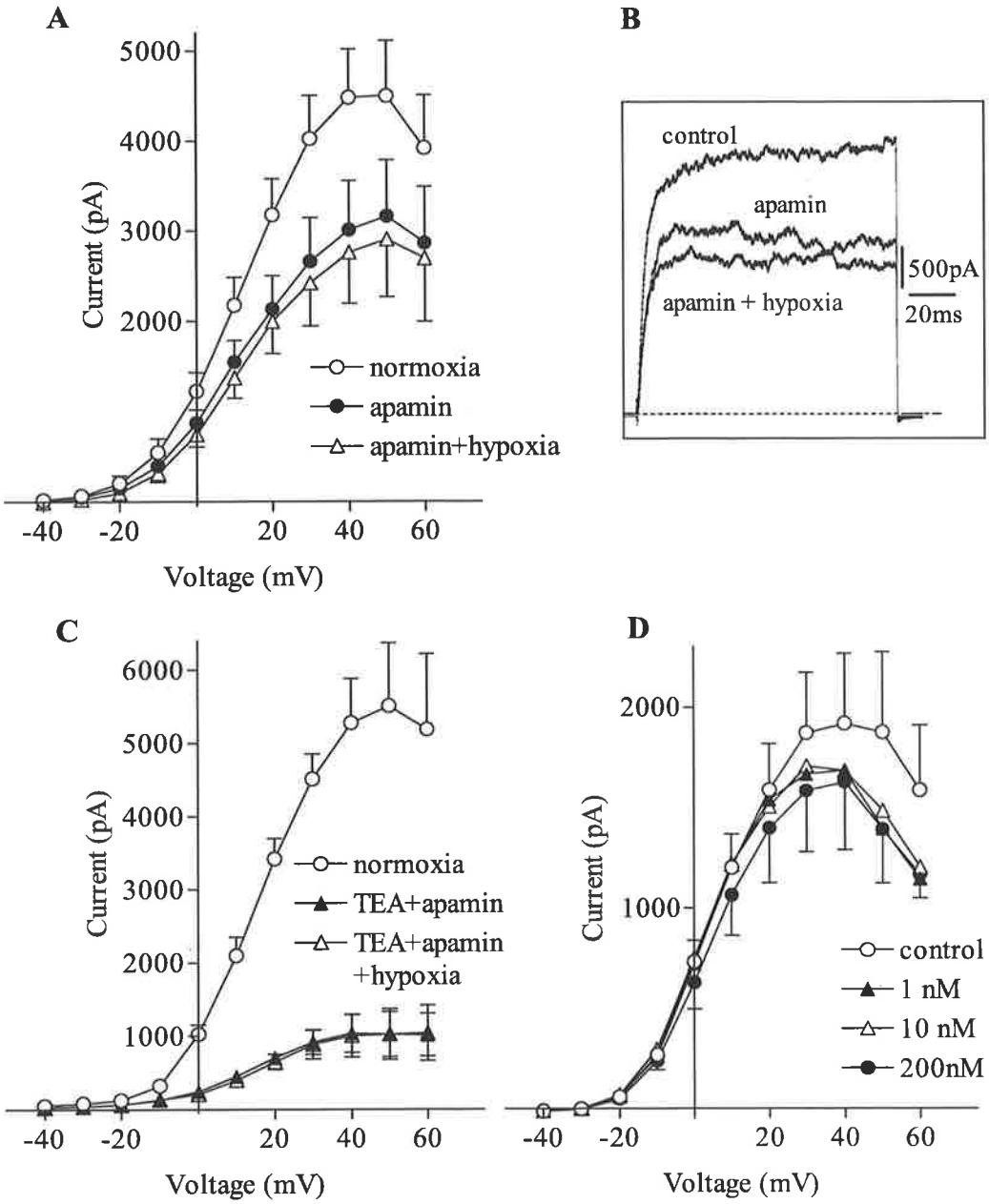

A
5000
4000
3000
2000
Current (pA)
normoxia
apamin
apamin+hypoxia
-40
-20
20
40
60
Voltage (mV)
B
control
apamin
apamin + hypoxia
500pA
20ms
C
6000
5000
4000
3000
2000
1000
Current (pA)
normoxia
TEA+apamin
TEA+apamin
+hypoxia
-40
-20
20
40
60
Voltage (mV)
D
2000
1000
Current (pA)
control
1 nM
10 nM
200nM
-40
-20
20
40
60
Voltage (mV)

Figure 2.2 (previous page): Contribution of apamin- and TEA-sensitive channels to oxygen sensitivity in fetal sheep AMCCs. **A** I-V relationship in control conditions (○) (n=9), in the presence of 200 nM apamin (n=9) (●), and during hypoxia in the presence of apamin (n=8) (◑) **B** Example trace of a whole cell current evoked by a single step pulse from -80 mV to +40mV during control, in the presence of 200 nM apamin and during hypoxia with apamin. **C** I-V relationship for K^+ current in normoxic conditions (○), in the presence of both apamin (200 nM) and TEA (1mM) (▲), and during hypoxia in the presence of both apamin and TEA (◑) (n=3). **D.** Sensitivity of fetal AMCCs to varying apamin concentrations. I-V relationship in control conditions (○) and in the presence of apamin at 1 nM (▲), 10 nM (◑) and 200 nM (●) (n=4). In all I-V plots, cells were held at -80 mV and stepped to potentials from -40 to +60 mV for 200 ms.

The availability of fetal sheep adrenal tissue is seasonal. Adult AMCCs are also capable of O_2-sensitivity once the influence of innervation has been removed (Adams *et al.*, 1996; Lee *et al.*, 2000). Consequently, these have been used for the later parts of this project, with initial experiments to confirm the identity of the channels responsible for the hypoxia-evoked reduction on K^+ currents in fetal and adult AMCCs.

Apamin (1 nM) significantly reduced Ca^{2+} -dependent K^+ currents in adult AMCCs in the range of 0 to 60mV (paired t-test; $p<0.05$; Fig 2.3). Hypoxia produced a reduction of a similar magnitude which was significant over the same range of membrane potentials ($p<0.05$). The application of hypoxia and apamin together gave a larger reduction in K^+ currents than was seen with apamin alone across a voltage range of 0 to 40 mV (paired t-test; $p<0.05$). There was overlap in the populations of channels blocked by

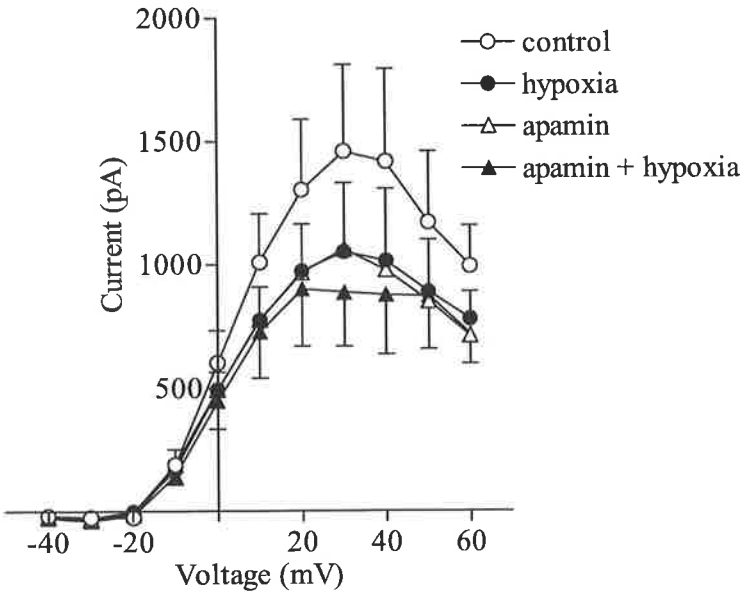
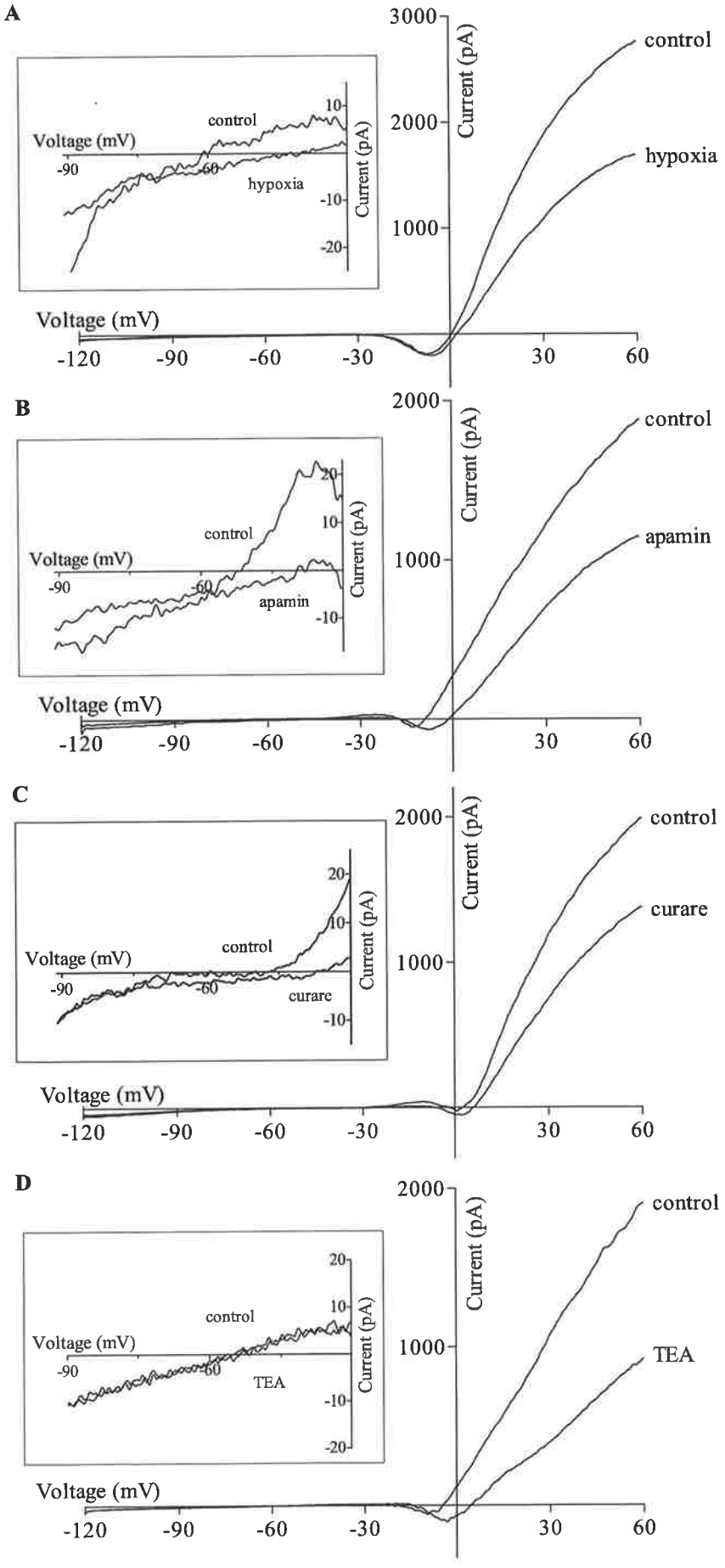


Figure 2.3: Contribution of apamin-sensitive channels to oxygen sensitivity in adult sheep AMCCs. I-V relationship in control conditions (○), during hypoxia (●), in the presence of 1 nM apamin (◑), and during hypoxia in the presence of apamin (▲) (n=5 for all). Cells were held at -80 mV and stepped to potentials ranging from -40 to +60 mV for 200 ms.

Figure 2.4 (following page): Example traces of the effect of **A** hypoxia, **B** 200 nM apamin, **C** 200 μM curare and **D** 1 mM TEA on whole cell currents in adult AMCCs occurring when cells are held at -60 mV and ramped from -120 to +60 mV over 100ms. Traces shown are the average of ten ramps under each condition. *Inset* magnification of current traces from -90 to -30 mV shows the shifting of reversal potentials (E_{rev}) due to hypoxia, apamin and curare.


A
3000
2000
1000
Current (pA)
control
hypoxia
Voltage (mV)
-120
-90
-60
-30
30
60
control
hypoxia
Voltage (mV)
-90
-60
10
-10
-20
Current (pA)
B
2000
1000
Current (pA)
control
apamin
Voltage (mV)
-120
-90
-60
-30
30
60
control
apamin
Voltage (mV)
-90
-60
20
10
-10
Current (pA)
C
2000
1000
Current (pA)
control
curare
Voltage (mV)
-120
-90
-60
-30
30
60
control
curare
Voltage (mV)
-90
-60
20
10
-10
Current (pA)
D
2000
1000
Current (pA)
control
TEA
Voltage (mV)
-120
-90
-60
-30
30
60
control
TEA
Voltage (mV)
-90
-60
20
10
-10
-20
Current (pA)

hypoxia and apamin since the reduction produced by the combined treatment (39 ± 6% at 30 mV) was less than the sum of the effects of hypoxia (28 ± 5 %) and apamin (27 ± 5%) alone. Thus in the adult, as in the fetus, the AMCCs have two classes of K_{O_2} currents, with SK channels accounting for a significant proportion of this.

2.3.4 Measurement of reversal potential

In order for K_{O_2} channels to participate in the initiation of the hypoxic response, closure of those channels must depolarise the cell membrane in order to stimulate Ca^{2+} influx and catecholamine secretion. A ramp protocol from -120mV to +60mV over 100ms from a holding potential of -60mV was used to investigate the effect of hypoxia, apamin and curare on the reversal potential (E_{rev}) of the membrane current of the adult AMCCs; E_{rev} can be used as a measure of the resting membrane potential. While the most obvious effect of all three treatments was seen at positive membrane potentials, each treatment also altered the reversal potential (Fig 2.4 A, B, C). The E_{rev} in control conditions was -55.1 ± 3.0 mV (n=18) and this shifted to more positive potentials during exposure to hypoxia (-46.4 ± 4.4 mV, n=5, $p<0.05$), apamin (-43.4 ± 5.1 mV, n=5, $p<0.05$) or curare (-44.7 ± 5.2 mV, n=8, $p<0.05$). These shifts in E_{rev} indicate that SK channels contribute to the resting potential of AMCCs and that closure of these channels by hypoxia or either of the blocking agents can cause depolarisation of the cell membrane.

In contrast to apamin and curare, 1 mM TEA failed to shift the E_{rev} of the I-V plot of the AMCCs (n=4) despite the significant block of the K^+ currents at more positive potentials (Fig 2.4D).

2.3.5 Intracellular Ca^{2+} measurements during hypoxia

The ability of membrane depolarisation, subsequent to closure of SK channels, to stimulate Ca^{2+} influx in sheep fetal AMCCs was investigated by the use of fluo-3 (Fig. 2.5A). Fluorescence intensity was increased by hypoxia (27 ± 4%, n=10; $p<0.05$) and by 200 nM apamin (24 ± 2%, n=7; $p<0.05$). The increase in $[Ca^{2+}]_i$, as indicated by the change in fluorescence, was not significantly different between cells treated with hypoxia or apamin. Simultaneous treatment with apamin and hypoxia produced an increase in fluorescence of 21 ± 5% (n=5) which was not significantly different from that produced by either treatment alone.

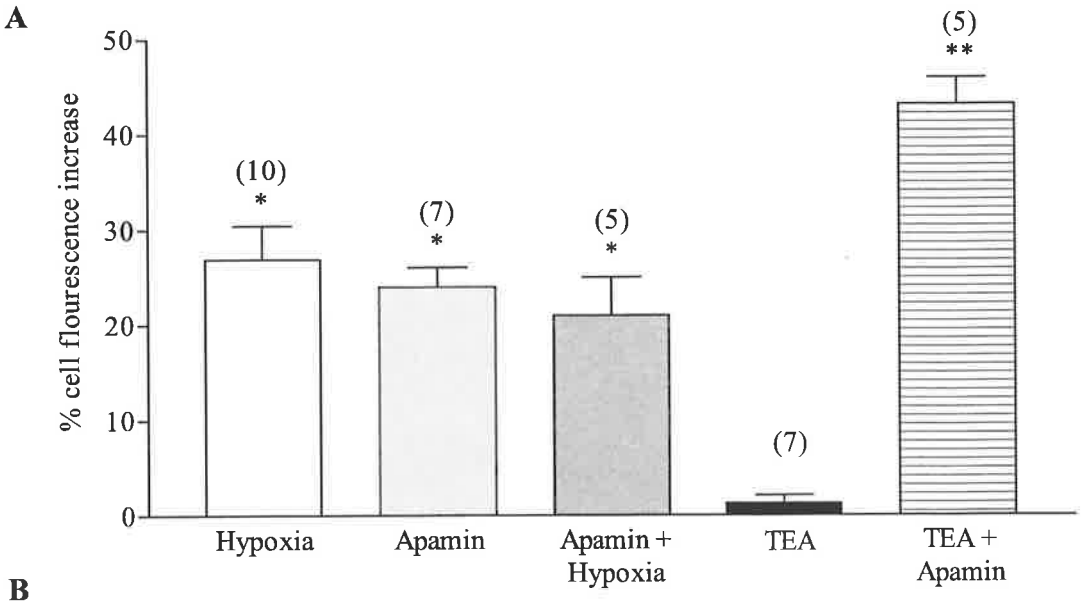
Similar results were seen in adult AMCCs (Fig. 2.5B) with equivalent increases in fluorescence produced by hypoxia (22 ± 4%, n=12), 200 nM apamin (24 ± 5%, n=7), and apamin and hypoxia simultaneously (21 ± 6%, n=5). The levels of fluorescence in these experimental conditions were all significantly greater than the fluorescence level in the control period ($p<0.05$), but were not different from each other. Curare (200 μM), another blocker of SK channels, caused a fluorescence increase of 23 ± 5% (n=7) while the combined curare and hypoxia caused a 26 ± 6% increase (n=7). There was no significant difference between the responses to curare, hypoxia or both of these combined.

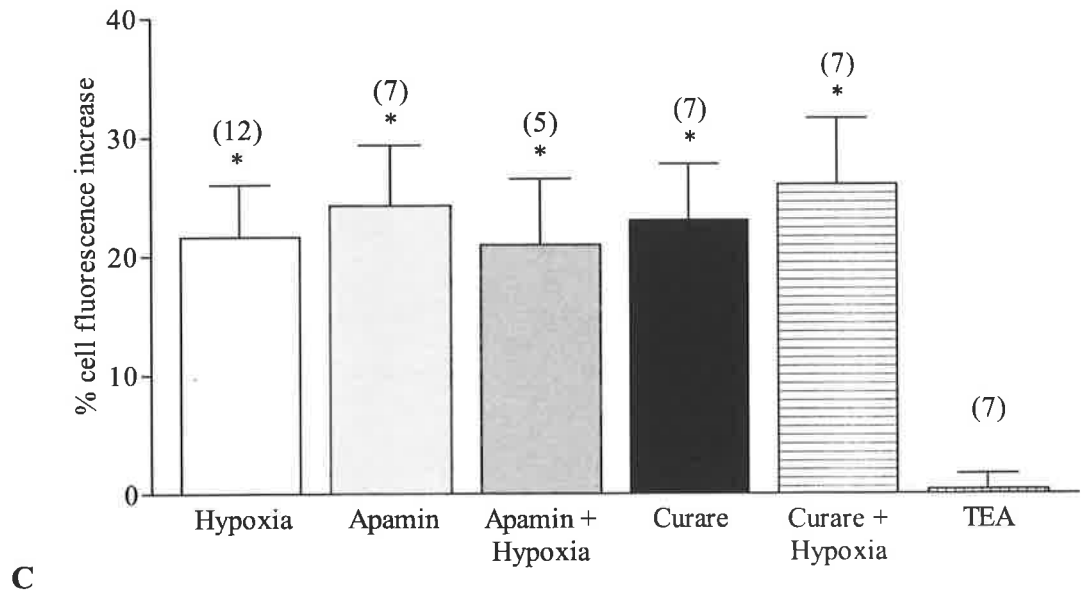
In view of the presence of an O_2-sensitive $K_{(Ca)}$ current in sheep AMCCs which can be blocked by TEA, as demonstrated in this study and previously (Rychkov *et al.*, 1998), the possible role of these channels in initiating the hypoxic response was investigated. TEA (10 mM) applied externally to both fetal and adult cells caused no significant increase in cell fluorescence (n=7). When TEA was applied along with apamin, the increase in cell fluorescence (43 ± 3%, n= 5) was significantly greater

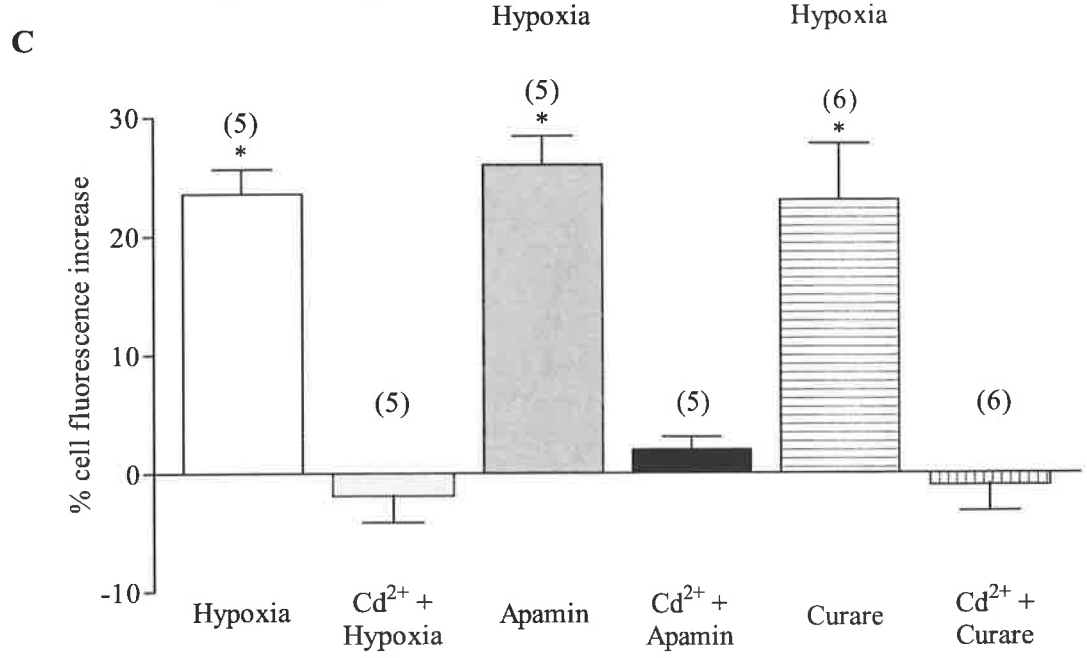
than that observed with apamin alone. Hence, it appears that although closure of TEA-sensitive channels can not depolarise the cell membrane to induce Ca^{2+} influx, it can modulate the cell response initiated by closure of apamin-sensitive K^+ channels.

To investigate the source of the increased $[Ca^{2+}]_i$ seen during acute hypoxia and upon the blockade of SK channels, Cd^{2+} (200 μM) was applied to the bath solution to block Ca^{2+} channels in the plasma membrane. In the presence of Cd^{2+}, exposure to hypoxia, apamin or curare did not induce a significant change in the fluorescence level, which indicates that normally these treatments stimulate Ca^{2+} entry from the extracellular space, but do not cause Ca^{2+} release from the intracellular Ca^{2+} stores (Fig. 2.5C).

Figure 2.5 (following page): Effects of hypoxia and K^+ channel blockers on intracellular Ca^{2+} in **A** fetal and **B** adult AMCCs. Fluorescence of fluo-3 in each cell, measured one minute after the application of various treatments, was compared to fluorescence in the control normoxic period. **C** The effect of hypoxia, apamin and curare on Ca^{2+} influx was blocked by application of 200 μM Cd^{2+}. Concentrations of drugs used in all graphs were 200 nM apamin, 200 μM curare and 10 mM TEA. The number of cells used for each treatment is shown in parenthesis. Single asterisks (*) indicate that these groups are significantly greater than the control values ($p<0.05$) but not different from each other. Double asterisks (**) indicate this group is significantly different from the control levels and all groups marked with a single asterisk ($p<0.05$).


A
% cell flourescence increase
50
40
30
20
10
0
(10)
*
(7)
*
(5)
*
(7)
(5)
**
Hypoxia
Apamin
Apamin + Hypoxia
TEA
TEA + Apamin

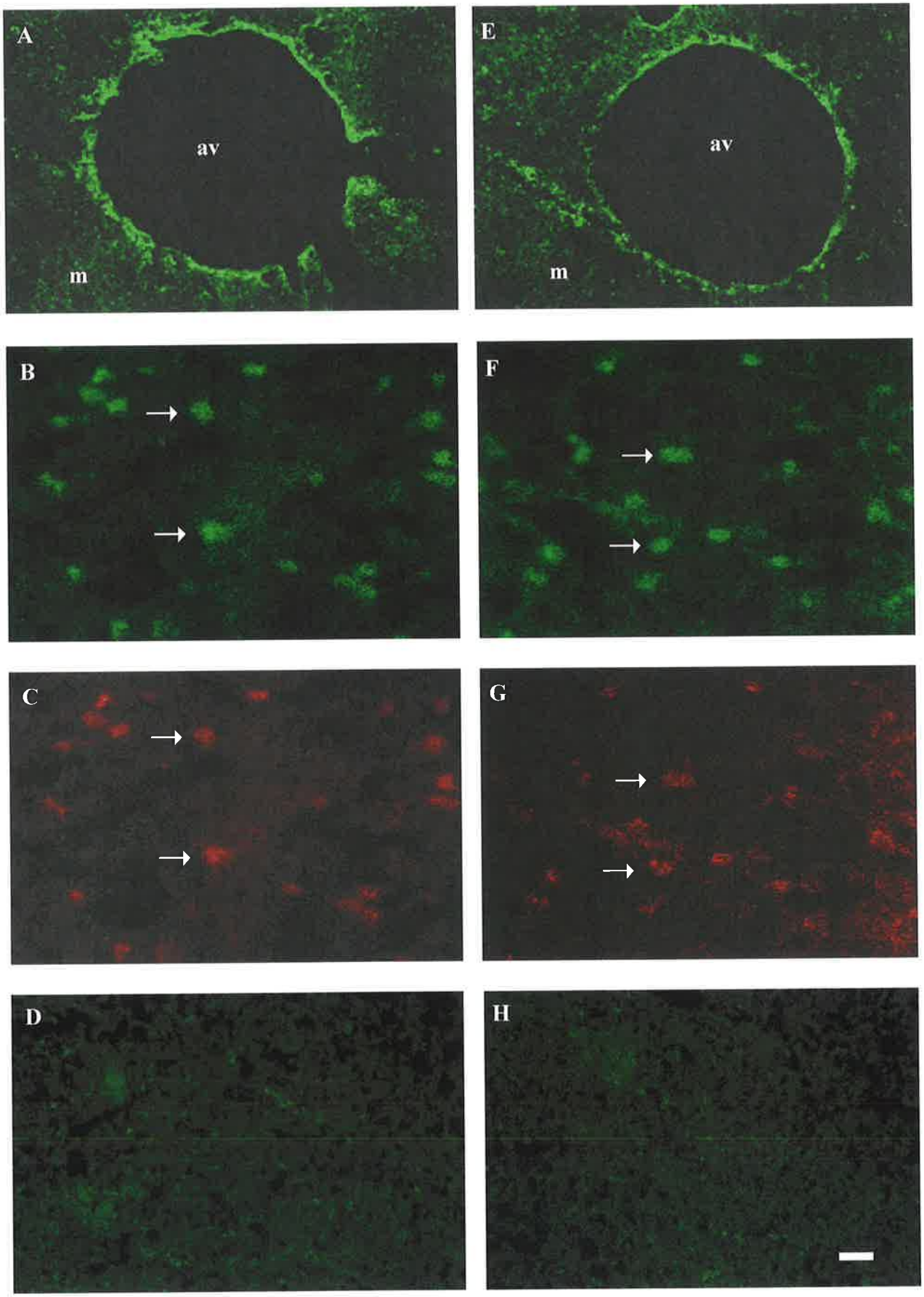

B
% cell fluorescence increase
40
30
20
10
0
(12)
*
(7)
*
(5)
*
(7)
*
(7)
*
(7)
Hypoxia
Apamin
Apamin + Hypoxia
Curare
Curare + Hypoxia
TEA


C
% cell fluorescence increase
30
20
10
0
-10
(5)
*
(5)
(5)
*
(5)
(6)
*
(6)
Hypoxia
Cd2+ + Hypoxia
Apamin
Cd2+ + Apamin
Curare
Cd2+ + Curare

2.3.6 Immunolocalisation of SK channels in the adult adrenal gland

Adrenal gland slices from adult sheep were stained with antibodies specific for both SK2 and SK3 channels. Staining for both the SK2 (Fig. 2.6A) and SK3 (Fig. 2.6E) channels is present throughout the adrenal medulla, but not in the adrenal cortex. Staining was greatest around the adrenal vein and other vascular areas of the adrenal medulla and no staining was seen in slices which had the relevant antigen pre-mixed with the antibody (Fig. 2.6D & H). Positive staining for TH occurred throughout the adrenal medulla but not in the adrenal cortex and, similarly to SK channel staining, was strongest in central medullary cells (results not shown). Almost all cells which stained positively for TH also stained positively for SK2 (Fig. 2.6B & C) or SK3 (Fig. 2.6F & G) indicating that SK2 and SK3 channels are located in catecholaminergic cells of the adrenal medulla. As SK2 and SK3 are both located in almost all TH-containing cells within the adrenal medulla, it reasonable to believe that these two K^+ channel types are located in the same population of cells as each other.

Figure 2.6 (following page): Immunolocalisation of SK2 and SK3 channels in the adrenal gland. Fluorescence images from adult adrenal gland slices indicate positive staining for SK2 channel (**A**) and SK3 channel (**E**). Cells were double labeled with TH to confirm that SK channels are localised in catecholamine-containing cells and this was confirmed with overlapping staining for both SK2 and TH (**B&C**) and SK3 and TH (**F&G**), examples indicated by the arrows. No immunofluorescence was seen when SK antibodies were pre-absorbed with their specific antigens as illustrated for SK2 (**D**) and SK3 (**H**). av = adrenal vein, m = adrenal medulla. Scale bar represents 500 μm in A and E, 50 μm in B, C, F and G and 200 μm in D and H.


A
av
m
E
av
m
B
F
C
G
D
H

2.4 Discussion

The ability of the fetal adrenal medulla to secrete catecholamines in response to hypoxaemia implies that the AMCCs possess an O_2 sensor. In the glomus cells of the carotid body, a major chemoreceptor in adult mammals, the role of the O_2 sensor is played by K_{O_2} channels that close in hypoxic conditions. It is well established that closure of K_{O_2} channels in glomus cells causes depolarisation of the cell membrane, resulting in Ca^{2+} influx into the cytosol and exocytosis of neurotransmitters (reviewed in Chapter 1). There are a number of different types of K^+ channels in glomus cells of different species that are inhibited by hypoxia. Moreover, a certain degree of variation exists even between different preparations within the same species. For example, in isolated glomus cells of the rat, closure of TASK-1-like K^+ channels by hypoxia is responsible for membrane depolarisation, and 10 mM TEA is ineffective in mimicking the hypoxic response (Buckler *et al.*, 2000). In rat carotid body slices, however, a Ca^{2+}-dependent BK channel is implicated as the K_{O_2} channel, and, in contrast to the previous study, application of 5 mM TEA depolarises the membrane, causing the release of catecholamines (Pardal *et al.*, 2000). In the rabbit carotid body, it has been suggested that closure of HERG-like channels that regulate resting potential may be responsible for the initial depolarisation (Overholt *et al.*, 2000) and that an O_2-sensitive transient K^+ current modulates the response (Ganfornina and Lopez-Barneo, 1992; Overholt *et al.*, 2000). In PC12 cells, TEA (1 mM) stimulates the secretion of catecholamines apparently through membrane depolarisation caused by the blockade of K^+ channels (Taylor and Peers, 1998). The oxygen-sensitive K^+ channel in this cell type has been identified as Kv1.2 from the *Shaker* subfamily of voltage-gated K^+ channels (Conforti and Millhorn, 1997).

The carotid body and the adrenal medulla share a common embryological origin in the neural crest and, not surprisingly, K_{O_2} channels have been proposed to act as the O_2 sensor in AMCCs of the adrenal medulla of the rat (Thompson *et al.*, 1997) and sheep (Rychkov *et al.*, 1998). Previous work in this laboratory has shown that the AMCCs of the fetal sheep possess K_{O_2} channels and application of 5 mM Co^{2+} revealed that all of this O_2-sensitive current is Ca^{2+}-dependent (Rychkov *et al.*, 1998). Ca^{2+}-dependent BK and SK channels have both been identified as O_2-sensitive in rat AMCCs. Thompson and Nurse (1998) reported that in neonatal rat AMCCs BK channels account for the major portion of the hypoxic suppression of outward current, however, block of BK channels by iberiotoxin failed to induce membrane depolarisation and did not affect the depolarisation caused by hypoxia. Studies on adult rat AMCCs implicate SK channels as the K_{O_2} channel (Lee *et al.*, 2000). Apamin, a selective inhibitor of SK channels, blocked all of the K_{O_2} current in these cells and caused depolarisation of the cell membrane. The effects of hypoxia and apamin were not additive.

In the present work O_2-sensitive BK and SK channels have both been found to be present in adult and fetal sheep AMCCs. In the presence of high concentrations of apamin (200 nM) that would block almost all SK channels, some O_2-sensitive current remained indicating that SK are not the only K_{O_2} channels in these cells. The fact that this remaining O_2-sensitive current was Ca^{2+}-dependent and was completely blocked by application of 1 mM TEA indicates that the channel mediating this current is the BK channel. Complete block of all O_2-sensitive currents by simultaneous application of apamin and TEA strongly suggests that SK and BK channels account for all of the K_{O_2} current in sheep AMCCs.

Of the apamin-sensitive SK channels, SK2 has a higher affinity for apamin (IC_{50} = 60 pM) than SK3 (IC_{50} = 1 nM) or SK1 (IC_{50} = 28 nM) (Kohler *et al.*, 1996; Ishi *et al.*, 1997; Ro *et al.*, 2001). In the study on adult rat AMCCs which reported the presence of an O_2-sensitive SK current, the very high concentrations of apamin (400 nM) used did not allow identification of the particular channel subtype involved (Lee *et al.*, 2000). In the fetal sheep AMCCs, 1 nM apamin produced a maximal effect, arguing that SK2 is most likely to be responsible for the apamin-sensitive current. This conclusion is supported by the analysis of mRNA in rat AMCCs where the mRNA for SK2, but not for SK3, has been found (Kohler *et al.*, 1996). The present results show that staining for both SK2 and SK3 antibodies is present in the adult adrenal medulla. Developmental differences in SK channel expression have not been eliminated however, and whether both these channels are expressed in the fetal adrenal medulla remains to be seen.

The colocalisation of TH with either SK channel indicates that these K^+ channels are located in catecholamine-containing AMCCs. The greatest number of SK- and TH-positive staining cells were found near the adrenal vein, which is similar to previous results which show that TH and phenylethanolamine N-methyl transferase (PNMT) expression in late gestation fetal and neonatal sheep adrenal medulla is high in both the peripheral and central areas of the medulla surrounding the adrenal vein (McMillen *et al.*, 1988; Riley *et al.*, 1992). Intense staining for enkephalin-containing peptides, which are co-released with catecholamines from AMCCs (Wilson *et al.*, 1982), is also apparent in the central adrenal medullary region of 80 to 120 days gestation fetal sheep (McMillen *et al.*, 1988) and these peptides have been implicated in suppressing the direct hypoxic response in AMCCs (see Chapter 4). This close

proximity to the circulation would enable O_2-sensitive cells containing SK channels to swiftly respond to changes in blood P_{O_2} levels.

Oxygen sensitivity of the K^+ currents by itself does not prove that they are involved in initiating the hypoxic response; in order for hypoxia to stimulate catecholamine secretion, closure of K_{O_2} channels must depolarise the cells and open voltage-dependent Ca^{2+} channels. In adult sheep AMCCs, hypoxia caused a significant shift in the reversal potential to more positive potentials. Similar results were seen with the application of either apamin or curare indicating that the closure of SK channels is capable of depolarising the cell membrane and initiating the hypoxic response. In contrast, TEA did not shift membrane potential of the sheep AMCC, suggesting that BK channels are closed at resting potential in these cells and are not involved in the membrane depolarisation caused by hypoxia.

These results are supported by measurements of cytosolic $[Ca^{2+}]$ in both the fetal and adult AMCCs. Since the increase in $[Ca^{2+}]_i$ produced by the SK blockers apamin and curare is the same as that produced by hypoxia, and the effects on $[Ca^{2+}]_i$ of either of these blockers in conjunction with hypoxia are not additive, closure of SK channels alone could explain the change in membrane potential resulting from hypoxia. Although TEA itself does not induce Ca^{2+} influx, the combination of TEA and apamin results in a much greater increase in $[Ca^{2+}]_i$ than that seen when apamin is applied alone. This, presumably, is a result of the prolongation of action potential duration when TEA-sensitive channels are blocked. This has been seen to occur in dorsal vagal neurones of the rat where blockade of SK channels causes increased action potential firing frequency while BK channel block results in increased action potential duration (Pedarzani *et al.*, 2000).

A very recent study has shown that steroid hormones which are produced in the adrenal cortex can alter the type of BK channel present in the adrenal medulla. Two splice variants of BK channels, named STREX and ZERO, are located in rat AMCCs (Xie and McCobb, 1998). Cortisol and the synthetic glucocorticoid, dexamethasone, decrease the ratio of STREX to ZERO expression while adrenal androgens increase this ratio (Lai and McCobb, 2002). When expressed in *Xenopus laevis* oocytes, these STREX BK currents activate at membrane potentials 20 mV more negative than ZERO currents and have faster activation and slower deactivation than ZERO currents (Xie and McCobb, 1998). It is conceivable then that, as dexamethasone is contained in the cell culture medium used for the experiments in the present study, the expression of STREX BK channels may be downregulated, thus, altering the function of BK channels in these AMCCs. If these STREX BK channels are open at the resting membrane potential and are expressed in the adrenal medulla *in utero*, the hypoxic suppression of BK current would result in membrane depolarisation and possibly catecholamine secretion. It is interesting that fetal sheep plasma cortisol levels increase from 125-130 days gestation (Norman *et al.*, 1985), the same gestational age as the onset of functional adrenal innervation (Cheung, 1990). This shift in the type of BK channel expressed in these cells could perhaps explain how the progressive reduction in the direct hypoxic response of the adrenal medulla occurs at the same time as the development of adrenal gland innervation *in utero*, but this is still a mechanism which remains purely hypothetical.

The reduction in $K_{(Ca)}$ current during exposure to hypoxic conditions could result from a hypoxia-evoked decline in Ca^{2+} influx, while on the other hand, direct increase of the Ca^{2+} currents by hypoxia could explain catecholamine release (see section 1.2.6 for review of O_2-sensitive Ca^{2+} channels). While there is some evidence

from the carotid body for a reduction in voltage-dependent Ca^{2+} current amplitude as a result of hypoxia (Lopez-Barneo *et al.*, 1997), more recent work has shown an increase in such currents (Summers *et al.*, 2000) in this tissue. In the present experiments, voltage-dependent Ca^{2+} currents were not affected by hypoxia, suggesting that the reduction in $K_{(Ca)}$ is not secondary to a reduced $[Ca^{2+}]_i$. This result also dismisses the possibility that the hypoxic secretory response in sheep AMCCs is initiated by an increase in Ca^{2+} current amplitude or a shift in their voltage dependence of activation to more negative potentials.

In summary, these results show that the direct response to hypoxia in AMCCs isolated from the sheep adrenal medulla, characterised by membrane depolarisation and a subsequent influx of Ca^{2+} through voltage-dependent Ca^{2+} channels, is initiated by closure of SK channels. BK channels in these cells are also O_2-sensitive but their likely role is to modulate the action potentials which result from closure of SK channels.

Involvement of $K_{(Ca)}$ channels in depolarisation of cell membrane due to hypoxia seems paradoxical, as the Ca^{2+} influx that results from a drop in P_{O_2} should activate more $K_{(Ca)}$ channels and cause cell hyperpolarisation. However these events are separated in time, with membrane depolarisation, Ca^{2+} influx and catecholamine release occuring first. A subsequent increase in $K_{(Ca)}$ currents due to an increase in $[Ca^{2+}]_i$ may provide an important negative feedback mechanism that protects AMCCs from Ca^{2+} overload in long periods of hypoxia.

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

3.1 Introduction

Many types of O_2 sensing cells exist amongst mammals including carotid body glomus cells, NEB, AMCCs, PASMC and PC-12 cells. Amongst these cells a common mechanism has appeared whereby a drop in P_{O_2} results in the closure of K_{O_2} channels, producing membrane depolarisation, an influx of Ca^{2+} ions into the cytosol and an increase in neurotransmitter or hormone secretion, or smooth muscle contraction, depending on the type of cell effected.

Although this K^+ channel closure during instances of low P_{O_2} is common throughout many O_2-sensitive cell types, the cellular events which occur between the stimulus of reduced O_2 availability and the effect of decreased channel open time, remains a point of conjecture. The most popular theory in O_2-sensitive cells is that of a redox model, in which a decrease in O_2 availability during hypoxia alters the enzymatic production of ROS such as O_2^- and H_2O_2. This, in turn, alters the redox state of the cell, possibly altering the function of ion channels which contain redox-sensitive amino acid groups. The two systems proposed as most likely to be responsible for these changes in ROS availability, and therefore the likely O_2 sensors, are NADPH oxidase and mitochondria.

The enzyme NADPH oxidase converts O_2 into the highly reactive superoxide anion which is quickly dismutated either spontaneously or enzymatically to H_2O_2 (Cross *et al.*, 1990; Acker, 1994). The involvement of NADPH oxidase in O_2 sensing was first suggested in the rat carotid body (Acker *et al.*, 1989) and also in fetal rabbit pulmonary NEB (Youngson *et al.*, 1993). In NEB, increased external H_2O_2 or activation of the oxidase with phorbol esters, potentiate K_{O_2} current amplitude (Wang *et al.*, 1996; O'Kelly *et al.*, 2000) and hypoxic sensitivity of these channels is

abolished in oxidase-deficient mice (Fu *et al.*, 2000). Normal hypoxic pulmonary vasoconstriction still occurs, however, in oxidase-deficient mice (Archer *et al.*, 1999). In the immortal NEB-derived cell line H-146, application of two structurally unrelated NADPH oxidase inhibitors decreases the hypoxic response only partially (O'Kelly *et al.*, 2001), providing the possibility of multiple O_2 sensing mechanisms existing in the one cell type.

The mitochondrion is an obvious choice for the O_2 sensor, as it consumes the majority of the available oxygen within a cell and is the major source of ROS owing to inefficient transport of electrons in the respiratory chain (Schultz and Chan, 2001). Evidence from some cell types implicate the mitochondria as the O_2 sensor, as rotenone, which halts the flow of electrons at complex I of the ETC, is able to block gene induction by hypoxia in a variety of O_2-sensitive cells (Semenza, 1999). Also rotenone, but not antimycin A, a blocker of complex II of the ETC, can impede the response to hypoxia in rat carotid body slices, signifying that a rotenone inhibitable molecule is involved in O_2 sensing in these cells (Pardal and Lopez-Barneo, 2000).

Few investigations have considered identifying the intracellular processes underlying O_2 sensitivity in AMCCs. Already in this thesis, Ca^{2+}-sensitive SK and BK channels have been identified as the K_{O_2} channels in ovine AMCCs, but how the function of these channels is mediated by changing O_2 tensions remains unknown. In order to study the possible role and sources of ROS which could be implicated in the hypoxic response of ovine AMCCs, whole cell patch clamp techniques have been employed to measure K^+ conductance changes during hypoxia, as well as in the presence of rotenone, H_2O_2 and the NADPH oxidase inhibitor, DPI.

3.2 Methods

3.2.1 Isolation of adrenal chromaffin cells

Isolated adult ovine adrenal glands were obtained from a local abattoirs and AMCCs were enzymatically dispersed as previously described in Section 2.2.1.

3.2.2 Electrophysiology

Whole-cell recordings were conducted according to the methods previously described in Section 2.2.2. The only variation from this was that whole cell K^+ currents were recorded using a pipette filling solution containing (mM): KCl, 48; K-glutamate, 75; $MgCl_2$, 2; MgATP, 5; $CaCl_2$, 0.66; EGTA, 1.2; HEPES, 10; NaGTP, 0.5; NaH_2PO_4, 1; Na-pyruvate, 2.5; malic acid, 2.5; adjusted to pH 7.2 with KOH.

3.2.3 Drugs

Rotenone, diphenylene iodonium (DPI) and hydrogen peroxide were all obtained from Sigma Chemicals, USA.

3.2.4 Statistics

Statistical comparisons were made using an ANOVA two factor statistical test with repeated measures and a Tukey's post hoc test to determine which groups significantly differed from each other. $p<0.05$ was taken as the minimum level of significance and results are expressed as means ± SEM.

3.3 Results

3.3.1 K^+ conductance changes during increased ROS exposure

The involvement of either the ETC or NADPH oxidase as O_2 sensors in the non-neurogenic hypoxic response has been hypothesised to occur via a reduced production of ROS such as O_2^- and H_2O_2. The effect of extracellular H_2O_2 on K^+ conductance and current size was tested (Fig. 3.1) using voltage ramps from -120 mV to 60 mV applied for 100 ms from a holding potential of -80 mV. The measurement taken from this ramp protocol was the chord conductance between the potentials of 10 mV and 50 mV (this measurement now to be referred to simply as chord conductance), a region of conductance which has previously been shown to be significantly altered during hypoxia (Section 2.3). H_2O_2 (200 μM) increased the chord conductance significantly from 33.5 ± 9.2 nS to 45.0 ± 11.3 nS ($p<0.05$). This effect was fully reversible upon washout with control solution, returning the chord conductance to a value of 31.8 ± 7.2 nS.

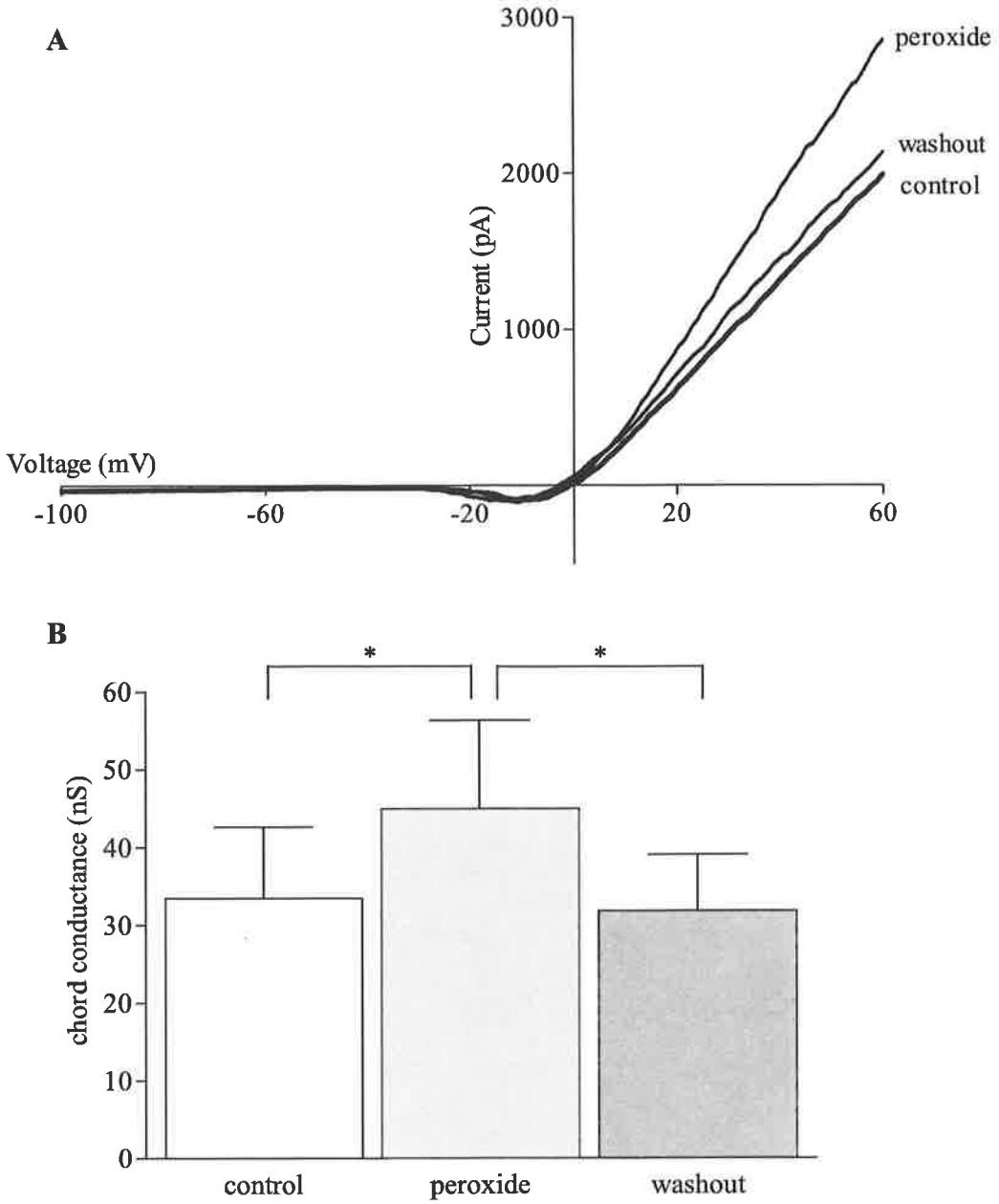


Figure 3.1: External H_2O_2 (200 μM) increases whole cell chord conductance. **A** Example current trace of a cell subjected to the ramp protocol described in Section 3.3.1. **B** the chord conductance between 10 mV and 50 mV was measured under various conditions. Asterisk indicates a significant difference between the two groups ($p<0.05$; $n=5$).

3.3.2 Involvement of NADPH oxidase in oxygen sensing

To look at the contribution that NADPH oxidase may have in the direct hypoxic response of adult AMCCs, DPI (20 μM) was applied to cells under both normoxic and hypoxic conditions during whole cell patch-clamp recordings (Fig. 3.2). The chord conductance under hypoxic conditions was significantly reduced from 38.4 ± 6.1 nS to 30.0 ± 6.5 nS ($p<0.02$) and this was fully reversed upon perfusion with normoxic solution to a value of 37.1 ± 5.6 nS. DPI also decreased chord conductance to a value of 29.6 ± 5.2 nS ($p<0.02$) and in a hypoxic environment, in the presence of DPI, this value was significantly reduced further to 21.5 ± 4.3 nS ($p<0.05$).

3.3.3 Role of mitochondria as the oxygen sensor

The association between the hypoxic response and the mitochondrial ETC was also investigated. This was achieved by disrupting the flow of electrons through the ETC with rotenone, a specific electron transport inhibitor acting at complex I. In this set of cells, hypoxia decreased the chord conductance from 35.6 ± 8.6 nS to 25.7 ± 6.7 nS ($p<0.05$; Fig. 3.3). These changes were fully reversed upon a return to normoxic conditions with chord conductance returning to 35.1 ± 8.9 nS. Rotenone (1 μM) had a similar effect to that of hypoxia by reducing chord conductance to 26.3 ± 6.5 nS ($p<0.05$) but in the presence of rotenone, hypoxia had no significant effect on chord conductance which remained at 26.5 ± 6.1 nS.

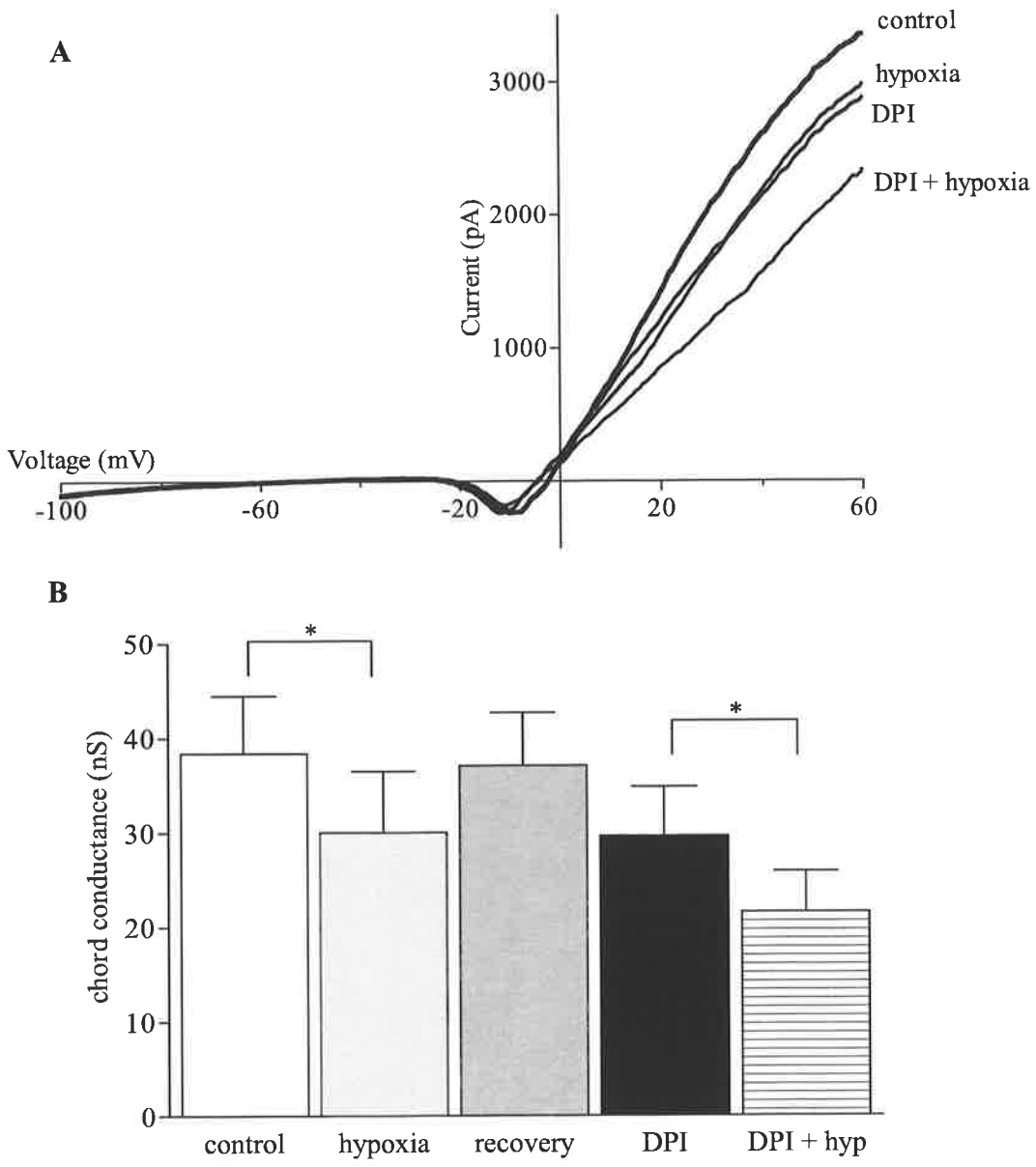


Figure 3.2: DPI (20 μM) has no significant effect on the hypoxic suppression of K^+ current. **A** Example current trace of a cell subjected to the ramp protocol described in Section 3.3.1. **B** chord conductance between 10 mV and 50 mV was measured under various conditions. Asterisk indicates a significant difference between the two groups ($p<0.05$; n=4).

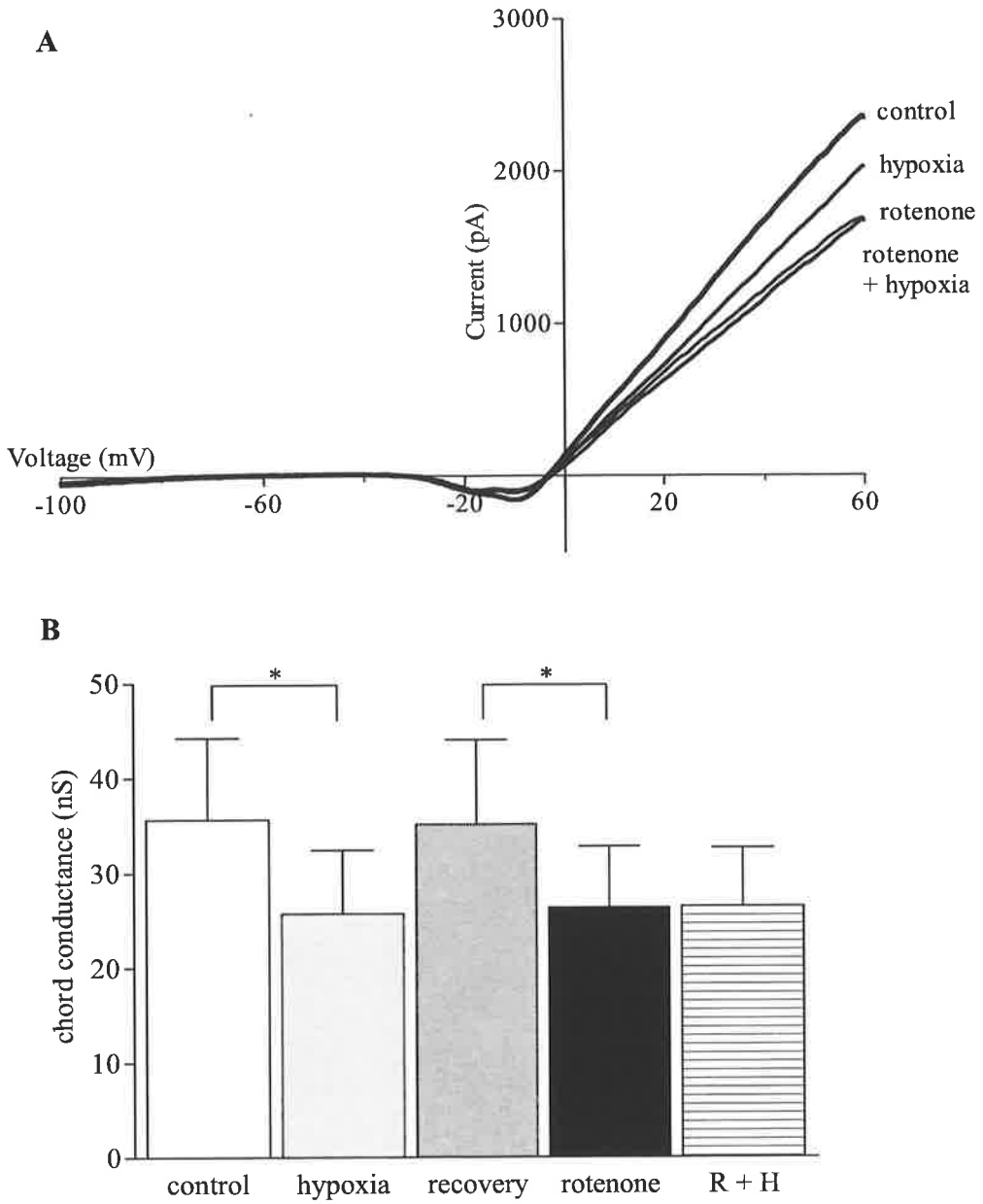


Figure 3.3: Rotenone (1 μM) application inhibits the hypoxic suppression of K^+ current in AMCCs. **A** Example current trace of a cell subjected to the ramp protocol described in Section 3.3.1. **B** Chord conductance between 10 mV and 50 mV was measured under various conditions. Asterisk indicates a significant difference between the two groups ($p<0.05$; n=4).

3.4 Discussion

This investigation began after it was observed that extracellular application of H_2O_2 clearly increases the K^+ current amplitude and conductance at positive membrane potentials, suggesting that alterations in redox state generate changes in ion channel kinetics in AMCCs. H_2O_2 is capable of rapidly diffusing across lipid membranes (Turrens and Boveris, 1980; Turrens *et al.*, 1985; Radi *et al.*, 1991) and hence, it seems plausible that extracellular H_2O_2 is entering the cell, oxidising the cytosol and increasing the opening of K^+ channels. This is in accordance with the redox-based hypothesis of how K_{O_2} channels could be modulated by changing P_{O_2} levels, which states that hypoxia induces a fall in cytosolic ROS levels, reducing the cytosol to cause K^+ channel closure. Previous experimental evidence in rat PC-12 cells has shown that H_2O_2 concentration decreases by approximately 40% during hypoxia, while hypoxic induction of tyrosine hydroxylase mRNA, the rate-limiting enzyme in catecholamine synthesis, is impeded by pretreatment with extracellular H_2O_2 (Kroll and Czyzyk-Krzeska, 1998). H_2O_2 production also decreases in rat carotid bodies during hypoxia and H_2O_2 scavengers reduce chemosensory discharge from this tissue (Acker *et al.*, 1992). A HERG-like current is active at, and responsible for the control of, resting membrane potential in rabbit glomus cells (Overholt *et al.*, 2000) and HERG currents expressed in two different cell systems are augmented upon an increase in intracellular ROS levels (Taglialatela *et al.*, 1997; Berube *et al.*, 2001). While this indicates that ROS may play a role in K^+ current modulation, the oxygen sensitivity of this HERG current has yet to be investigated.

Mitochondria and NADPH oxidase have been regularly implicated in O_2 sensing as they are both intracellular sites of ROS generation. It appears that the

inhibition of NADPH oxidase function by DPI does not inhibit the normal hypoxic suppression of K^+ conductance seen in adult sheep AMCCs. This is in agreement with rat and rabbit carotid body preparations, where NADPH oxidase inhibition does not alter hypoxia-induced catecholamine secretion (Obeso *et al.*, 1999), and in genetically mutated NADPH oxidase-deficient mice, which retain an unaffected level of HPV in their pulmonary arteries (Archer *et al.*, 1999).

The lack of contribution to the hypoxic suppression of K^+ current by NADPH oxidase in AMCCs, the carotid body and pulmonary arteries is in contrast to the mechanism of O_2 sensing which occurs in NEB cells. In cultured fetal and neonatal rabbit NEB cells, K^+ current is increased by H_2O_2 and the hypoxic suppression of K^+ current does not occur in the presence of DPI (Wang *et al.*, 1996; Fu *et al.*, 1999). This K^+ current inhibition in NEB by hypoxia and DPI, is also seen in wild-type, but not oxidase-deficient gene knock-out, mice (Fu *et al.*, 2000). In line with the theory of redox modulation of K_{O_2} channels, the current augmentation by H_2O_2 which is seen in wild-type mice remains in oxidase-deficient mice (Fu *et al.*, 2000).

These results indicate that, while NADPH oxidase does not play a role as the O_2 sensor in sheep AMCCs, this oxidase does appear to be present due to the decrease in K^+ conductance caused by DPI. This, however, is not necessarily the case. While DPI has long been known to be an inhibitor of NADPH oxidase (Hancock and Jones, 1987; O'Donnell *et al.*, 1993), it also inhibits several flavoproteins (O'Donnell *et al.*, 1994) and is a non-selective blocker of ion channels (Weir *et al.*, 1994; Lopez-Barneo *et al.*, 2001). It is therefore unclear whether the effect of DPI seen in our cells are due to inhibition of NADPH oxidase, non-selective ion channel blockade, or a combination of the two. One definite outcome of these experiments is that NADPH

oxidase can be ruled out as the O_2 sensor in sheep AMCCs, as hypoxia still causes a decrease in K^+ conductance in the presence of DPI.

Limiting mitochondrial electron flow impedes the hypoxia-induced reduction of K^+ conductance in sheep AMCCs, implying a mitochondrial role in O_2 sensing. This is in line with results regarding HPV in intrapulmonary arteries, in which HPV is abolished by inhibition of complex I or III, with rotenone or myxothiazol, respectively (Leach *et al.*, 2001). In NADPH oxidase-deficient mice, vasoconstriction still occurs due to rotenone application (Archer *et al.*, 1999), consistent with a role for the ETC in O_2 sensing in PASMC. In rat AMCCs, hypoxia, rotenone and cyanide all increase cytosolic $[Ca^{2+}]_i$ and catecholamine secretion, with simultaneous application of hypoxia and cyanide producing the same effect as either stimulant alone, indicating a related mechanism of action (Mojet *et al.*, 1997). Our results in sheep AMCCs are in accordance with these results across a variety of O_2 sensing tissues and illustrate that mitochondria are capable of acting as the intracellular O_2 sensor.

The hypotheses that ROS production modulates channel activity during hypoxia also presumes that the ratio of intracellular redox pairs may be altered, in turn causing possible modifications to disulfide bonds of K^+ channel proteins. One example of a redox pair which has been investigated for its role during hypoxia is the ratio of reduced to oxidised glutathione (GSH/GSSG). In fast-inactivating RCK4 K^+ channels, cysteine residues in the N-terminus of the channel have been revealed as being responsible for the increased channel inactivation which occurs when the intracellular environment is reduced by GSH (Ruppersberg *et al.*, 1991). Cysteine-specific oxidising agents also effect inactivation properties of cloned rat Kv1.4 channels by removing inactivation, slowing deactivation and increasing the voltage-sensitivity of deactivation (Stephens *et al.*, 1996). The GSH/GSSG ratio is postulated

to change during hypoxia in PASMC (Archer *et al.*, 2000), as hypoxia and GSH both reduce K^+ currents in these cells, while GSSG increases K^+ current amplitude (Park *et al.*, 1995).

Methionine residues, similarly to cysteine, are also susceptible to oxidation in a reversible manner *in vivo*, a process regulated through the specific actions of the enzyme methionine sulfoxide reductase (MSR). This oxidation can have functional implications for methionine-containing ion channels, such as *Shaker* K^+ channels, in which channel inactivation is disrupted upon oxidation of a N-terminus methionine residue to methionine sulfoxide by either the oxidant chloramine-T (Ciorba *et al.*, 1997) or NO (Ciorba *et al.*, 1999), an effect reversed in the presence of MSR. It is possible that this enzyme has a similar impact on K^+ channels in AMCCs, as MSR has been cloned from the bovine adrenal medulla (Ciorba *et al.*, 1997). The effect of hypoxia on reducing methionine residues and altering K^+ channel structure in cells containing MSR may be minimal, as these amino acids are likely to already be in a reduced state during normoxia. The association between methionine modification and ion channel structure and function in oxygen-sensitive cells is, however, a topic which requires a great deal of further investigation before any real conclusions can be made.

Apamin-sensitive SK channels are responsible for causing the membrane depolarisation which leads to opening of VGCCs and increased $[Ca^{2+}]_i$ during hypoxia. All SK channels contain at least nine cysteine and twenty methionine residues, several of them located in the N-terminus, near the pore forming region and in the calmodulin binding site which is required for channel gating. These properties of SK channels make redox modification of this K_{O_2} channel during hypoxia a feasible possibility.

Our results indicate that in adult dispersed ovine AMCCs, the mitochondrial ETC plays a role as the O_2 sensor. Further work remains to be carried out in the use of cloned SK channels and their O_2 sensitivity and the possible effect that point mutations of cysteine and methionine residues could have on channel gating during changes in redox state. This will allow the hypothesis that SK channel gating is altered by redox state due to changes in thiol residues to be thoroughly investigated and shed further light on the mechanism by which AMCCs respond to hypoxia. This hypothesis has gained further support from recent work in our laboratory which has found that currents produced by heterologously expressed apamin-sensitive SK2 and SK3, but not apamin-insensitive SK1 channels, are decreased in amplitude by the reducing agent DTT (personal communication P. Giacomin, 2002).

4. μ- AND κ-TYPE OPIOID RECEPTOR STIMULATION SUPPRESSES THE DIRECT HYPOXIC RESPONSE IN OVINE ADRENAL CHROMAFFIN CELLS

4.1 Introduction

The direct response of AMCCs to hypoxia is seen in the mammalian fetus during early gestation, before the development of the innervation of the adrenal gland (Cheung, 1990), where hypoxaemia-evoked elevations in circulating catecholamines are crucial for fetal survival (Cheung, 1990). After splanchnic innervation has developed, the direct non-neurogenic response of the adrenal medulla to hypoxia is suppressed, and hypoxia-evoked catecholamine secretion occurs as a result of a neurogenic chemoreceptor reflex originating in the carotid body (Seidler and Slotkin, 1986b; Cheung, 1990). Adrenal denervation restores the direct response of the AMCCs to hypoxia (Cheung, 1990), indicating that a factor released from the nerve terminals may be responsible for the suppression of the non-neurogenic response. Nerve terminals in the adrenal medulla release a number of biologically active agents including acetylcholine, PACAP, NO, VIP and endogenous opioid peptides (Kobayashi *et al.*, 1985; Holgert *et al.*, 1998; Tornoe *et al.*, 2000). The action of acetylcholine on nicotinic receptors is not responsible for the suppression of the non-neurogenic response of the AMCCs to hypoxia, as pre-treatment with the nicotinic antagonist hexamethonium does not restore the non-neurogenic response in sheep (Cheung, 1990).

The potential for various neurotransmitters released from the splanchnic nerves to suppress the direct hypoxic response has been discussed in detail in Section 1.4. It appears that of these various neurotransmitters, endogenous opioid peptides have actions which are best suited for a role in suppressing the direct hypoxic response. All three major subclasses of opioid receptor, μ, δ, and κ, have been identified in the bovine adrenal medulla (Dumont and Lemaire, 1984; Bunn *et al.*,

1988). Opioid peptides are present in the nerve terminals of the adrenal medulla (Kobayashi *et al.*, 1985) and are also co-secreted with catecholamines from AMCCs during nicotinic stimulation (Livett *et al.*, 1981). Opioid agonists have been reported to increase the current through BK channels (Twitchell and Rane, 1993), decrease voltage-dependent Ca^{2+} currents (Kleppisch *et al.*, 1992; Albillos *et al.*, 1996a) and inhibit the secretion of catecholamines (Saiani and Guidotti, 1982) in single bovine AMCCs. As well as this, in the late gestational fetal sheep, when the adrenal medulla would be innervated, the opioid antagonist naloxone potentiates the secretion of adrenaline evoked by acute hypoxaemia (Martinez *et al.*, 1988).

These effects of opioid receptor stimulation on BK channels and VGCCs would explain how opioid peptides released from the splanchnic nerve terminals reduce the release of catecholamines in response to a depolarizing stimulus, such as hypoxia, but they would not explain the elimination of the hypoxic response. Suppression of hypoxia-evoked secretion, in sheep AMCCs, would occur if opioids could prevent the hypoxia-evoked depolarisation produced by closure of SK channels, but there is no previous evidence of this occurring. Preliminary work in our laboratory has found that μ- and κ-type opioid receptor activation suppresses the hypoxia-evoked secretion of catecholamines from whole perfused fetal sheep adrenal glands (M. Adams, G. Rychkov and H. Holgert, unpublished data, see Appendix 1). The present study aims to determine whether opioids prevent hypoxia-evoked catecholamine secretion by the elimination of membrane depolarisation and subsequent Ca^{2+} entry caused by the closure of SK channels.

4.2 Methods

4.2.1 Isolation of adrenal chromaffin cells

Adult merino ewes were used for these experiments and AMCCs were enzymatically prepared as described in Section 2.2.1.

4.2.2 Electrophysiology

Whole-cell recordings were conducted as described in Section 2.2.2. The only variation from this was that when recording whole cell K^+ currents with $[Ca^{2+}]_i$ clamped, the extracellular solution was the same as that described in Section 2.2.2 with the addition of $CdCl_2$ (0.2 mM) and the pipette filling solution contained (mM): KCl, 150; MgATP, 1; EGTA, 1; HEPES, 10; $CaCl_2$, 0.89; adjusted to pH 7.2 with KOH. The Ca^{2+} concentration in this internal solution was calculated to be 1μM (Eqcal, Biosoft, Cambridge, UK). Potassium conductance was estimated between 10 mV to 60 mV using voltage ramps which increased linearly from -120 mV to 100 mV over 100 ms from a holding potential of -80 mV.

When Ca^{2+} currents were recorded, the extracellular and intracellular solutions were the same as used for measuring Ca^{2+} currents described in Section 2.2.2. The opioid agonists [D-Arg^2, Lys^4]-dermorphin-(1-4)-amide H-Tyr-DArg-Phe-Lys-NH_2 (DALDA), [D-$Pen^{2,5}$]-enkephalin (DPDPE) and (±)-(5alpha,7alpha,8beta)-3,4-Dichloro-N-methyl-N-(7-[1-pyrrolidinyl]-1-oxaspiro[4.5]dec-8-yl) benzeneacetamidemesylate (U-62066), all at 1 μM, were applied through the superfusion solution for 3 to 5 minutes.

4.2.3 Calcium Imaging

Calcium imaging of single adult chromaffin cells was performed according to the methods outlined in Section 2.2.3.

4.2.4 Drugs

TEA, Apamin extracted from bee venom, NPPB and DMEM were obtained from Sigma Chemicals, USA, Fluo-3 AM and Pluronic F-127 from Molecular Probes, USA and the μ-agonist DALDA, δ-agonist DPDPE and κ-agonist U-62066 from RBI Chemicals, USA.

4.2.5 Statistics

Results are expressed as means ± SEM. For the confocal imaging data Student's paired t-test was used to compare differences of $[Ca^{2+}]_i$ after hypoxia with or without the presence of a specific opioid agonist as well as when comparing the effect of these agonists on peak Ca^{2+} current amplitude. Student's paired t-test and an analysis of variance (ANOVA) with Tukey's post hoc test were used for two group and multiple group comparisons, respectively, when chord conductance was measured from whole cell patch clamp data. A probability level of 5% ($p<0.05$) was taken as significant for all analyses.

4.3 Results

4.3.1 Opioid agonists and hypoxia-evoked changes in Ca^{2+} entry into AMCCs

All single cell studies used chromaffin cells isolated from the adrenal medulla of adult sheep. After one minute exposure to hypoxia, the $[Ca^{2+}]_i$ increased significantly, as indicated by an increase in fluorescence of fluo-3 loaded cells of 20.0 ± 2.9% ($p<0.0001$; Fig 4.1). In the presence of the μ-type agonist DALDA, while hypoxia evoked an increase in fluorescence (5.9 ± 1.5%; $n=7$; $p<0.01$), this was significantly smaller than that seen during hypoxia in the absence of this opioid agonist ($p<0.005$; Fig 4.1).

Stimulation of κ-type opioid receptors also reduced the change in $[Ca^{2+}]_i$ produced by hypoxia (Fig 4.1). In the presence of U-62066 (1 μM), the change in fluorescence produced by hypoxia was reduced from 22.2 ± 4.1% to 13.5 ± 1.9% ($n=5$; $p<0.05$). Stimulation of δ-type opioid receptors did not alter the change in $[Ca^{2+}]_i$ evoked by exposure to hypoxia (Fig 4.1).

The effects of opioid agonists on voltage-dependent Ca^{2+} currents were determined by the whole-cell patch clamp technique (Fig 4.2). Cells were treated with DALDA, DPDPE or U-62066 (1 μM) separately for approximately 3-5 minutes. The μ agonist, DALDA (Fig 4.2B), and the κ agonist, U-62066, (Fig 4.2C) decreased the Ca^{2+} current amplitude at 5 mV by approximately 17% ($p<0.05$) and 20% ($p<0.01$) respectively. Neither opioid agonist affected inward current at potentials more negative than -10 mV. DPDPE had no effect on Ca^{2+} current amplitude at any voltage (Fig. 4.2D). The inhibitory effect of the μ and κ opioid agonists on Ca^{2+} current was fully reversible upon washing with control solution.

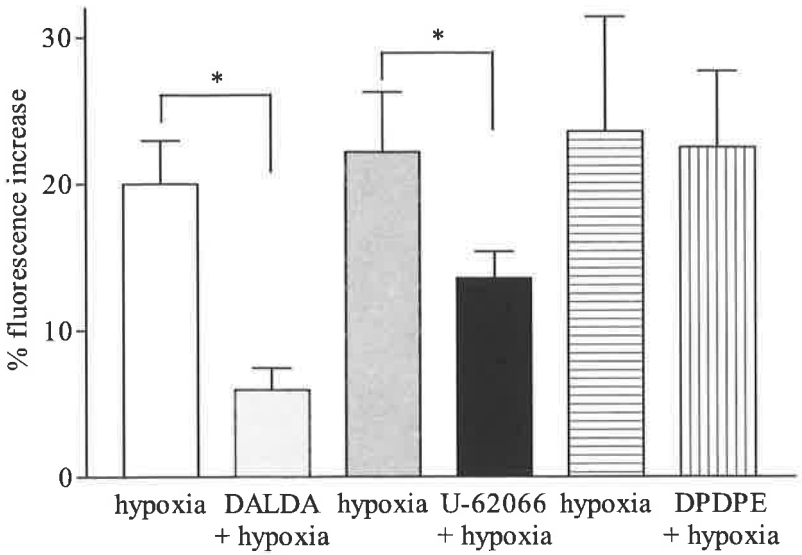


Figure 4.1: Effects of hypoxia on intracellular [Ca^{2+}] in the presence and absence of opioid receptor agonists. Fluorescence of fluo-3 in each cell, measured 1 minute after the application of various treatments, was compared to fluorescence in the control normoxic period. Different numbers of cells were used for results obtained from DALDA (n=7), U-62066 (n=5) and DPDPE (n=5). All values shown are significantly greater than the control fluorescence level during normoxia. * significantly different from the respective hypoxia value without the opioid agonist present ($p<0.05$).

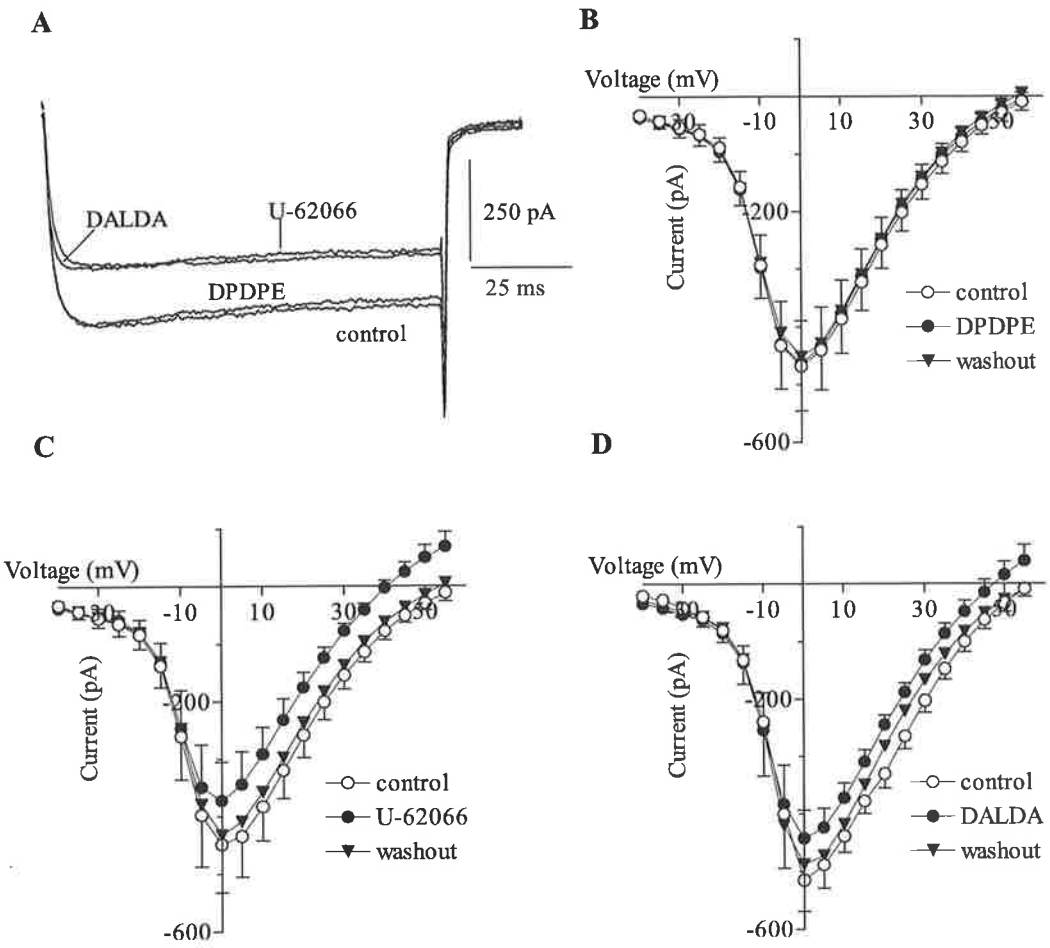


Figure 4.2: Calcium currents are inhibited by μ- and κ- opioid agonists but not by δ-opioid agonists. **A** Example trace of a Ca^{2+} current evoked by a single 100 ms step pulse from -80 mV to +5 mV in control conditions and in the presence of 1 μM of either DALDA, U-62066 or DPDPE. **B, C, D** The I-V relationship of Ca^{2+} current during control (○), with specific opioid receptor agonist (●), and upon washout with control solution (▼). The opioid agonist involved in each figure is DPDPE (B, n=6), U-62066 (C, n=6) and DALDA (D, n=4). Cells were held at -80 mV and stepped to potentials ranging from -40 to +55 mV for 100 ms in increments of 5 mV.

4.3.2 Opioid agonists and K^+ currents

As μ and κ agonists reduce the amplitude of whole-cell Ca^{2+} currents and $K_{(Ca)}$ currents are known to be present in sheep AMCCs, a method had to be found which allows the investigation of these agonists on K^+ currents without the simultaneous effects of Ca^{2+} current reduction. Therefore, the cytosolic Ca^{2+} concentration clamped at 1 μM by the use of a Ca^{2+}-buffered solution intracellularly and the addition of a Ca^{2+} channel blocker, Cd^{2+} (200 μM), to the extracellular solution.

At positive membrane potentials both μ and κ-type receptor stimulation cause significant changes in K^+ conductance (Fig. 4.3). Application of DALDA increased the slope conductance by 35% ($p<0.05$), while U-62066 decreased the slope conductance by 28% ($p<0.05$). The δ agonist DPDPE had no effect on K^+ conductance. Apamin (1 nM), a blocker of SK channels, and TEA (1 mM), a blocker of BK channels, were used to determine how these channels are effected by the changes in membrane conductance produced by μ and κ agonists. The increase in conductance produced by DALDA did not occur in the presence of apamin, indicating that the μ agonist acted on SK channels (Fig 4.4). In contrast, the decrease in conductance produced by U-62066 was not reduced by the application of apamin (Fig 4.5) but treatment of the cells with TEA abolished the ability of the κ agonist to reduce conductance, indicating an action on BK channels (Fig 4.6).

In physiological conditions, the effects of the μ and κ opioid agonists on Ca^{2+} currents and $K_{(Ca)}$ currents will interact. In order to determine the balance between these effects, K^+ currents were measured in cells in which the $[Ca^{2+}]_i$ was not clamped during the depolarizing pulses. Under these conditions, the μ agonist DALDA caused

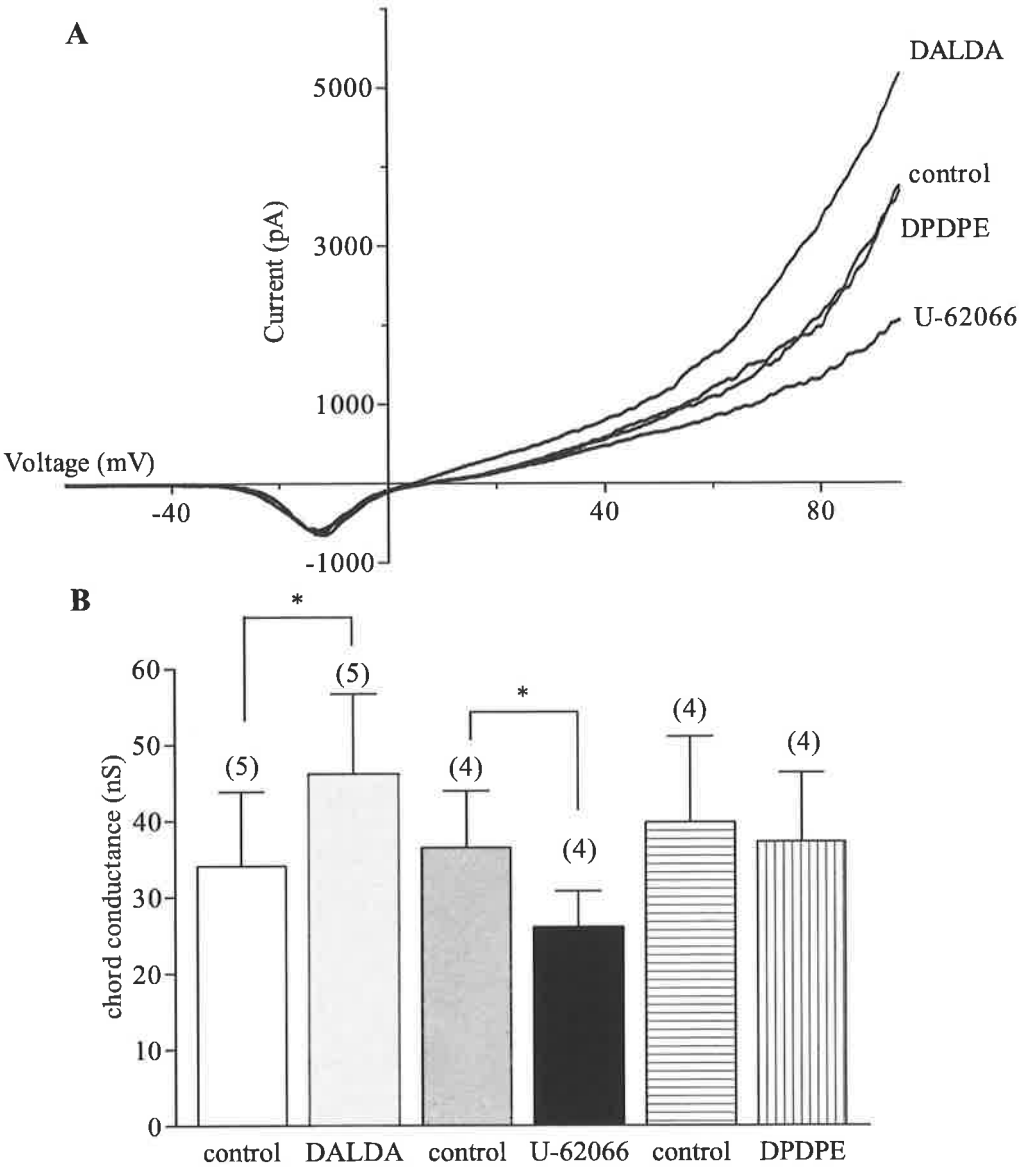


Figure 4.3: Effect of opioid agonists on K^+ conductance in AMCCs. **A** Example traces of opioid agonists effect on K^+ currents in adult AMCCs when cells are held at -80 mV and ramped from -120 to +100 mV over 100ms. Traces shown are the average of ten ramps under each condition. **B** Chord conductance of AMCCs measured in the positive voltage range of 10 mV to 60 mV. The number of cells used for each treatment is shown in parenthesis. * significantly different from the bracketed value ($p<0.05$).

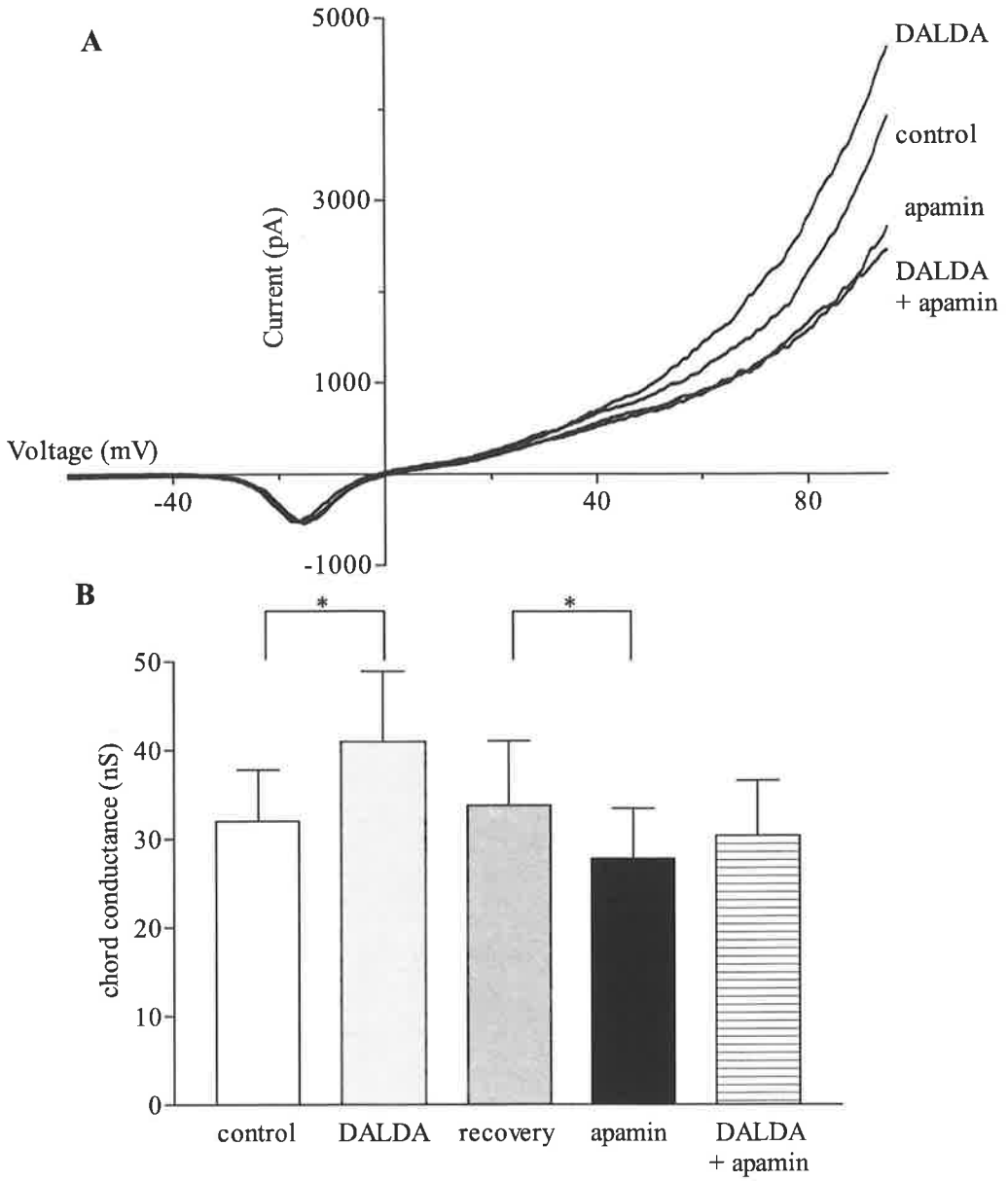


Figure 4.4: μ-type receptor stimulation increases SK channel conductance. **A** Example traces of the effect of DALDA (1 μM), apamin (1 nM) and both together on K^+ currents in adult AMCCs when cells are held at -80 mV and ramped from -120 to +100 mV over 100ms. Traces shown are the average of ten ramps under each condition. **B** Chord conductance of AMCCs measured in the positive voltage range of 10 mV to 60 mV (n=7). * significantly different from the bracketed value ($p<0.05$).

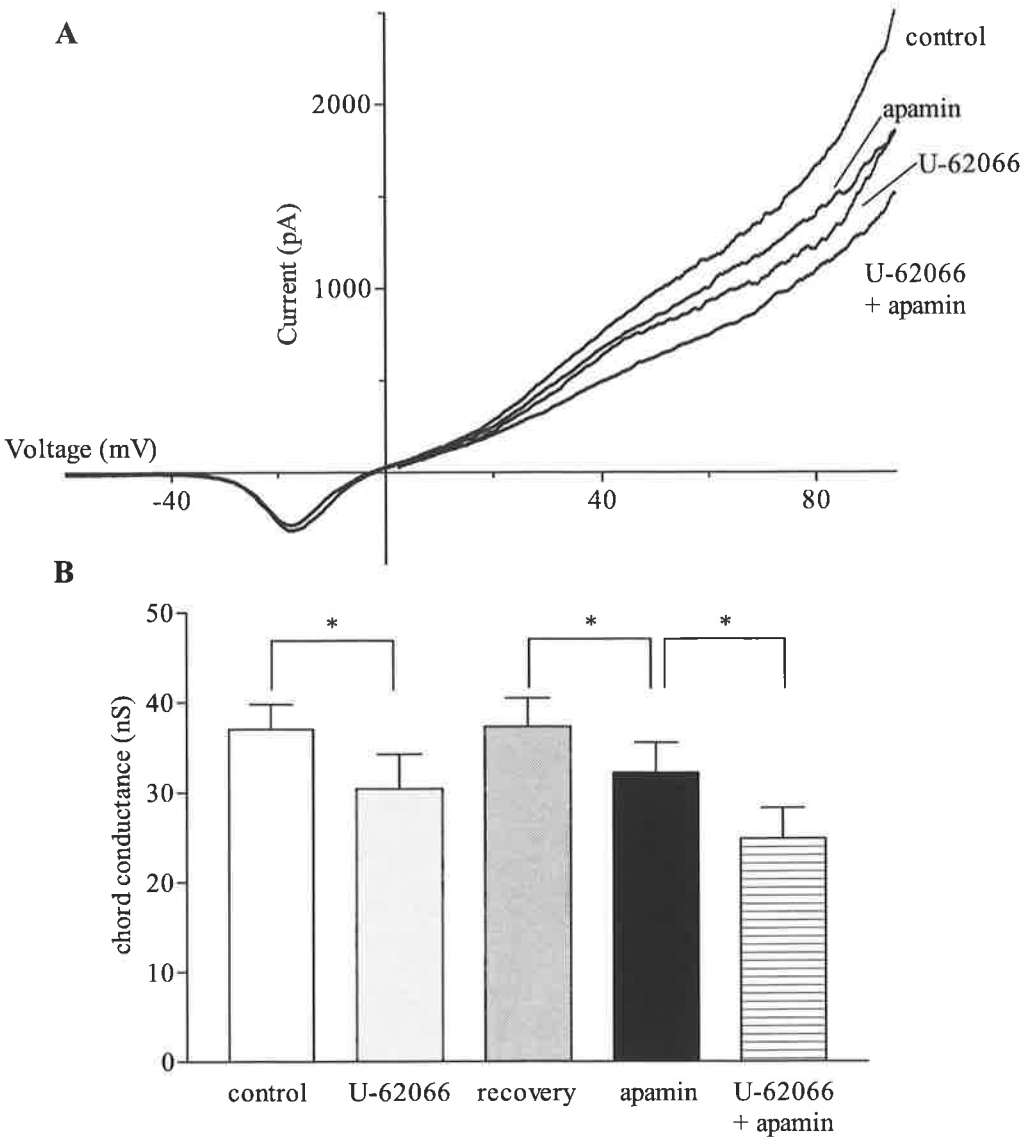


Figure 4.5: SK channel blockade does not interfere with the effect of κ-opioid receptor stimulation on K^+ conductance. **A** Example traces of the effect of U-62066 (1 μM), apamin (1 nM) and both together on K^+ currents in adult AMCCs when cells are held at -80 mV and ramped from -120 to +100 mV over 100ms. Traces shown are the average of ten ramps under each condition. **B** Chord conductance measured in the positive voltage range from 10 mV to 60 mV, n=4 for all columns. * significantly different from the bracketed value ($p<0.05$).

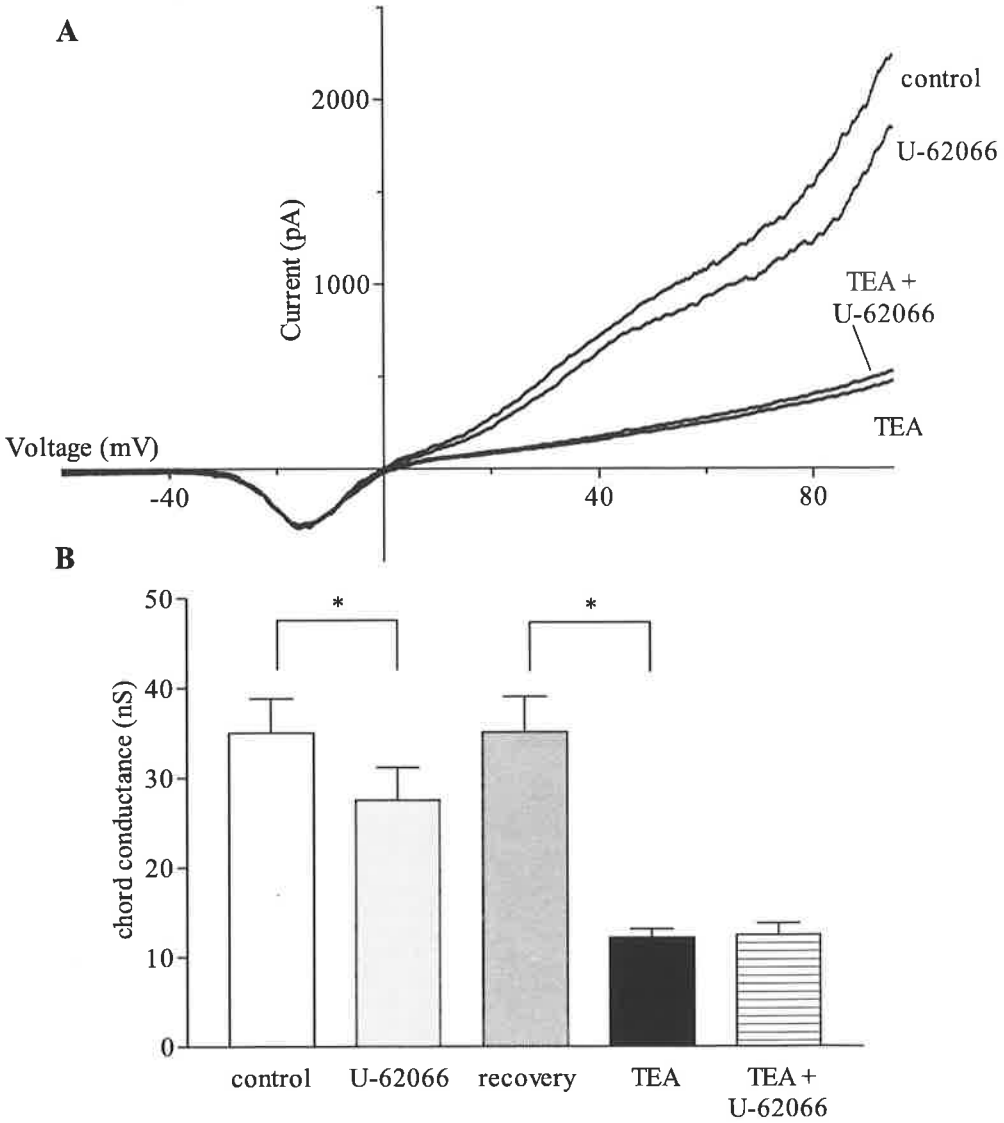


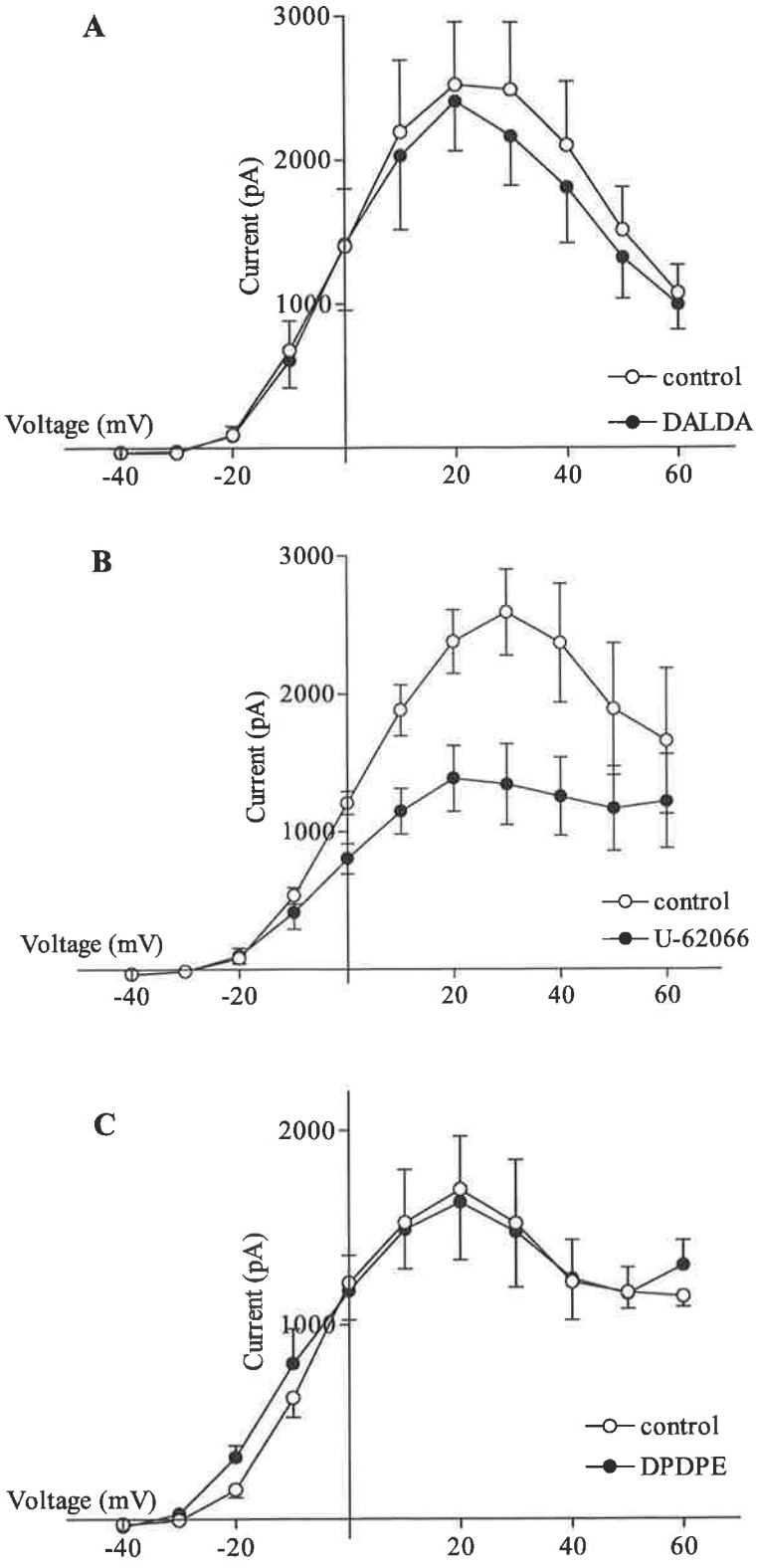
Figure 4.6: TEA inhibits the effect of κ-opioid receptor stimulation on K^+ conductance. **A** Example traces of the effect of U-62066 (1 μM), TEA (1 mM) and both together on K^+ currents in adult AMCCs when cells are held at -80 mV and ramped from -120 to +100 mV over 100ms. Traces shown are the average of ten ramps under each condition. **B** Chord conductance of AMCCs measured in the positive voltage range from 10 mV to 60 mV, n=5 for all columns. * significantly different from the bracketed value ($p<0.05$).

no significant change in outward current at any potential (n=5; Fig 4.7A). Stimulation of κ opioid receptors with U-62066 produced a significant reduction in outward current at membrane potentials between 0 and 50 mV (n=5; $p<0.05$; Fig 4.7B). Stimulation with the δ-type opioid agonist DPDPE had no effect on outward current in these cells (Fig 4.7C).

4.3.3 Effect of μ opioid agonist on the hypoxia-evoked reduction of K^+ current

In this group of cells, where $[Ca^{2+}]_i$ was clamped at 1 μM, the ability of hypoxia to reduce overall membrane conductance was offset by the effects of DALDA (Fig 4.8). Hypoxia decreased membrane conductance by 6.1 ± 1.2 nS from the value of 32.2 ± 5.2 nS seen in normoxic conditions. DALDA did not inhibit the ability of hypoxia to close K^+ channels, as shown by the 6.6 ± 1.8 nS reduction in conductance produced by hypoxia in the presence of DALDA. The membrane conductance in the presence of both agents, 33.8 ± 6.4 nS, was, however, not different from that seen in control, normoxic cells.

Figure 4.7 (following page): Modulation of whole cell current by specific opioid agonists in adult sheep AMCCs. I-V relationship in control conditions (○) and in the presence of an agonist (●). Different receptor type agonists were used, all at 1 μM concentration including **A** DALDA (n=5), **B** DPDPE (n=4) and **C** U-62066 (n=5). Cells were held at -80 mV and stepped to potentials ranging from -40 to +60 mV for 200 ms.


A
3000
2000
1000
Current (pA)
control
DALDA
Voltage (mV)
-40
-20
20
40
60
B
3000
2000
1000
Current (pA)
control
U-62066
Voltage (mV)
-40
-20
20
40
60
C
2000
1000
Current (pA)
control
DPDPE
Voltage (mV)
-40
-20
20
40
60

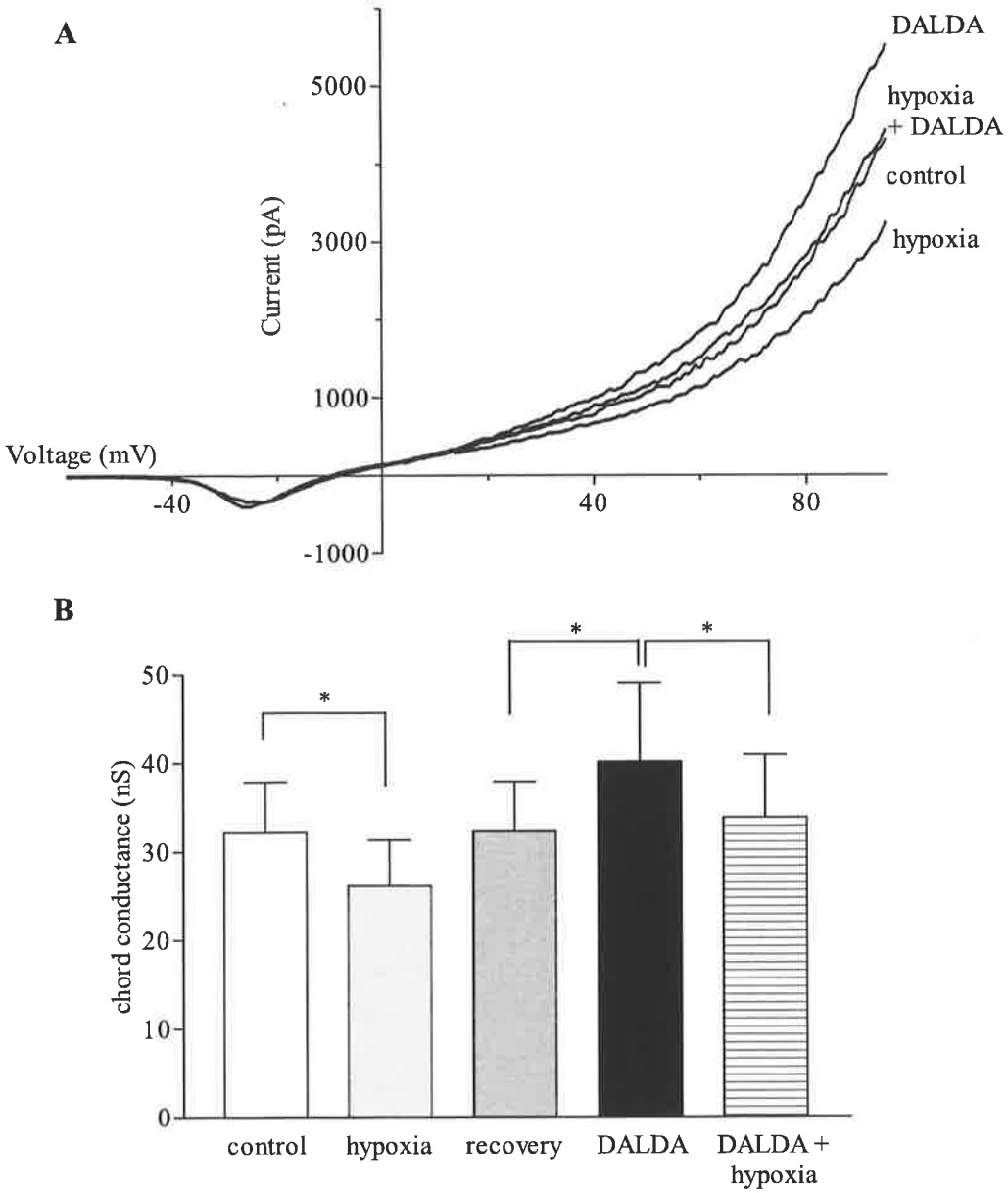


Figure 4.8: The effects of μ-opioid receptor stimulation and hypoxia on K^+ conductance. **A** Example traces of the effect of hypoxia, 1 μM DALDA and both together on K^+ currents in adult AMCCs when cells are held at -80 mV and ramped from -120 to +100 mV over 100ms. Traces shown are the average of ten ramps under each condition. **B** Chord conductance measured in the positive voltage range from 10 mV to 60 mV, n=5 for all columns. * significantly different from the bracketed value ($p<0.05$).

4.4 Discussion

In this study we have shown that opioid agonists, in addition to their previously described actions on currents through VGCCs and BK channels in bovine AMCCs (Kleppisch *et al.*, 1992; Twitchell and Rane, 1993; Albillos *et al.*, 1996a), also increase the current through SK channels in isolated sheep AMCCs. SK channels are important determinants of resting membrane potential in these cells as hypoxia reduces the opening of these channels, causing membrane depolarisation, Ca^{2+} entry and catecholamine secretion (Adams *et al.*, 1996; Rychkov *et al.*, 1998; Keating *et al.*, 2001). By its actions on SK channels, the μ agonist DALDA can offset the decrease in K^+ conductance produced by hypoxia, and significantly reduce the hypoxia-evoked increase in cytosolic $[Ca^{2+}]$.

While the adrenal medulla resembles a number of other tissues in the mechanism by which it responds directly to hypoxia (Adams *et al.*, 1996; Rychkov *et al.*, 1998; Keating *et al.*, 2001), it differs in that this direct hypoxic response is suppressed in the intact adrenal gland by splanchnic innervation (Cheung, 1990). The reappearance of the direct response of sheep AMCCs to episodes of acute hypoxia within a few hours of denervation, as seen previously *in vivo* (Cheung, 1990), in the perfused isolated whole adrenal gland (Adams *et al.*, 1996) and in isolated AMCCs (Rychkov *et al.*, 1998), indicates that the neural suppression is likely to be due to a neuromodulator released from the splanchnic nerve terminals. It has been proposed that this modulator may act to reduce the excitability of AMCCs (Butler *et al.*, 1995). There are several potential modulators released from these nerves but most of them have effects that could not explain suppression of the direct hypoxic response. Opioid peptides, which are known to be present in splanchnic nerve terminals (Kobayashi *et*

al., 1985) as well as in AMCCs (Livett *et al.*, 1981; Wilson *et al.*, 1982), have been shown to inhibit the release of catecholamines in bovine and canine AMCCs (Saiani and Guidotti, 1982; Kimura *et al.*, 1988) and therefore could possibly modulate the hypoxic response in the adrenal medulla.

In a study carried out in parallel with those described in this chapter, the combined stimulation of μ- and κ-type opioid receptors by their respective agonists, DALDA and U-62066, inhibited the hypoxia-evoked secretion of catecholamines from the isolated perfused adrenal gland of the fetal sheep (see Appendix 1). While this work demonstrated that opioid receptor stimulation significantly diminished hypoxia-evoked non-neurogenic catecholamine secretion from the fetal adrenal gland, the cellular mechanisms underlying this phenomenon remained unidentified. As catecholamine secretion is dependent on the influx of Ca^{2+} ions into the cytoplasm to trigger it, the effect of opioid agonists on hypoxia-evoked Ca^{2+} entry in AMCCs was investigated.

In these and further experiments, single adult AMCCs were utilised as these cells respond to hypoxia via the same mechanisms which occur in fetal AMCCs (refer to Section 2.3). The suppression of the hypoxia-evoked secretion by the opioid agonists appears to result from a reduction in $[Ca^{2+}]_i$, produced by stimulation of the μ and κ opioid receptors, during episodes of hypoxia. The δ agonist, DPDPE, had no effect on the hypoxia-evoked increase in $[Ca^{2+}]_i$. This would not appear to be a result of using a low concentration of DPDPE as the concentration used is over one hundred times greater than either the K_D for binding in rat brain membranes (Schlosser *et al.*, 1995) or the IC_{50} for blocking the electrically-stimulated mouse vas deferens (Haaseth *et al.*, 1990).

The influx of Ca^{2+} produced by hypoxia in AMCCs occurs through voltage-dependent Ca^{2+} channels (refer to Section 2.3). The decrease in Ca^{2+} currents in sheep AMCCs stimulated by μ and κ opioid agonists supports earlier work in bovine AMCCs where μ agonists decrease Ca^{2+} current (Albillos *et al.*, 1996a) and in neurones of the guinea pig myenteric plexus where κ agonists decrease Ca^{2+} channel conductance (Cherubini and North, 1985). Consistent with the current experiments in which $[Ca^{2+}]_i$ was measured, DPDPE did not affect voltage-dependent Ca^{2+} currents. The reduction in Ca^{2+} currents that was produced by μ and κ opioid agonists in the sheep AMCCs was, however, small, particularly in the case of the μ agonist, and would not appear to account for all of the large reduction in $[Ca^{2+}]_i$ that is seen when these agonists are present during hypoxic episodes.

In excitable cells such as AMCCs, Ca^{2+} entry through voltage-dependent Ca^{2+} channels is modulated by the activity of K^+ channels, which are also known to be regulated by opioid agonists (Twitchell and Rane, 1993). O_2-sensitive SK and BK channels play a central role in the direct hypoxic response of the adrenal medulla and are potential targets for opioid action. As the activity of both SK and BK channels are regulated by $[Ca^{2+}]_i$, any action of opioids agonists on these channels could be either direct on the channel or indirect through a reduction in Ca^{2+} current amplitude. In order to determine any direct effect of the opioid agonists on the oxygen-sensitive K^+ currents, measurements were made with the $[Ca^{2+}]_i$ clamped at 1 μM. Under these conditions, application of the μ-type opioid agonist DALDA increased the K^+ conductance of the isolated AMCCs. Inhibition of the DALDA-induced increase in membrane conductance by 1 nM apamin indicates that the μ agonist is acting on SK channels. This activation of SK channels by DALDA offsets the action of hypoxia on

these channels, with the consequence of this being that, in cells that are not voltage-clamped, μ-type opioid receptor stimulation will inhibit the depolarisation normally produced by hypoxia. This inhibition of hypoxia-evoked membrane depolarisation would also explain the mechanism by which DALDA reduces both Ca^{2+} influx and catecholamine secretion during hypoxia.

Activation of κ-type opioid receptors with U-62066 decreased the K^+ conductance of AMCCs. While apamin did not alter this effect of U-62066, TEA (1 mM), a blocker of BK channels, completely abolished the κ-type agonist-induced reduction in K^+ current. Although there are no previous reports of κ-type receptors reducing K^+ currents in AMCCs, it has been shown to occur in a catecholaminergic cell line (Baraban *et al.*, 1995). In sheep AMCCs it is the SK channel, rather than the BK channel, which is important for initiating the hypoxia-evoked secretion of catecholamines (refer to Section 2.3). This would indicate that suppression of the TEA-sensitive BK current by κ-type opioid receptor stimulation does not cause a membrane depolarisation that would counteract the membrane hyperpolarisation caused by μ-type receptor activation.

As well as the more direct effect of these agonists on the BK and SK channels, the activity of the calcium-dependent K^+ channels in these cells will be affected by the opioid-evoked reduction of Ca^{2+} currents under physiological conditions where $[Ca^{2+}]_i$ is not clamped to a set concentration. The balance between these two effects was seen in the whole-cell currents measured in cells in which $[Ca^{2+}]_i$ was not clamped. The outward current amplitude was the same in the presence and absence of the μ agonist, DALDA. The absence of any effect by this opioid agonist reflects the interaction between an increase in K^+ conductance through a direct action on the SK channels and

a reduction in SK and BK channel conductance resulting from the μ agonist-induced reduction in Ca^{2+} currents. With U-62066, the decrease in the outward current at positive potentials would reflect a combination of the reduction in BK conductance produced by the direct action of κ agonist on these channels and the indirect action on BK and SK channels caused by the reduction in voltage-dependent Ca^{2+} currents.

With regard to the direct hypoxic response of AMCCs, we have shown previously that closure of SK channels initiates depolarisation, leading to Ca^{2+} entry, which would trigger catecholamine secretion. At potentials near resting membrane potential, the sole effect of the opioid agonists would be an increase in K^+ conductance resulting from the direct action of the μ receptor on SK channels as voltage-dependent Ca^{2+} channels and BK channels in these cells are closed at these potentials and would be unaffected by the opioid agonists.

Previous studies have shown that endogenous opioids are active in the adrenal medulla during acute hypoxia as, in chronically catheterised fetal sheep, the opioid antagonist naloxone potentiates the hypoxia-evoked secretion of adrenaline, a response which appears to be due to inhibition of μ-type opioid receptors (Martinez *et al.*, 1988). From these investigations we have found a plausible explanation in the action of opioid peptides for the suppression of the non-neurogenic hypoxic response of the adrenal medulla upon innervation. It appears that both μ- and κ-type receptors play a role in this mechanism with stimulation of both receptor types reducing the opening of VGCCs, thus decreasing the level of hypoxia-evoked Ca^{2+} entry, while μ-type receptor activation increases the K^+ conductance attributed to SK channels.

5. THE CONTRIBUTION OF VGCCs TO Ca^{2+} ENTRY AND ACTIVATION OF Ca^{2+}-DEPENDENT K^+ CHANNELS IN FETAL AND ADULT CHROMAFFIN CELLS

5.1 Introduction

As has been pointed out a number of times in this thesis, developmental differences underlie the manner in which the adrenal medulla releases catecholamines in response to acute hypoxia *in vivo*. In the mature mammal, a reflex mechanism is utilised, but this reflex response does not occur in the fetus prior to the development of adrenal innervation in which case a non-neurogenic processes is utilised in order for the adrenal medulla to release catecholamines in response to hypoxia (Slotkin and Seidler, 1988; Cheung, 1990). At the early stages of this project, it was proposed that the disappearance of the direct hypoxic response could be a result of changes in the expression of ion channels in the AMCC membrane during development, and in particular, upon the onset of adrenal innervation.

Catecholamine release is a process which is highly Ca^{2+}-dependent (Knight, 1986), with Ca^{2+} entering cells primarily through VGCCs located in the cell membrane. These VGCCs are not only involved in catecholamine secretion but are also important in controlling the level of $K_{(Ca)}$ channel activation. There are six major subtypes of VGCCs identified so far: L-, N-, P-, Q-, R- and T-type (Fisher and Bourque, 2001). These channels, reviewed in Chapter 1, can be distinguished by certain properties such as their activation voltage, rates of inactivation and pharmacological blockade (Fisher and Bourque, 2001).

Due to the reliance of catecholamine secretion on Ca^{2+} entry through VGCCs, the ontogeny of Ca^{2+} channels present in the adrenal medulla could underlie the developmental differences in the response of this tissue to hypoxia. Hypoxia-evoked secretion preferentially involves L- and N-type Ca^{2+} channels in rat AMCCs (Mochizuki-Oda, 1997) and L-type Ca^{2+} channels in whole perfused sheep adrenal

glands (Adams *et al.*, 1996), while in PC-12 cells, N-type Ca^{2+} channels (Taylor and Peers, 1998) and L-type Ca^{2+} channels (Kumar *et al.*, 1998) have both been implicated as being preferentially involved in hypoxia-induced Ca^{2+} entry. The variance between these two studies with PC-12 cells may be due to differences in the age of the PC-12 cells used, as high external $[K^+]$-induced secretion occurs mainly through N-type Ca^{2+} channels in cells obtained within the past year from the American Type Culture Collection, and mainly L-type Ca^{2+} channels in cells frozen in storage for approximately 8 years (Green *et al.*, 2001).

As the hypoxia-evoked suppression of K^+ current in O_2-sensitive AMCCs causes Ca^{2+} entry through VGCCs and catecholamine secretion, the question arises of whether there is colocalisation of K^+ channels with certain Ca^{2+} channels. Colocalisation of these channels would be beneficial by reducing the time between the signal (membrane depolarisation) and the response (opening of VGCCs). It has been shown in isolated frog hair cells that activation of $K_{(Ca)}$ channels upon voltage-dependent Ca^{2+} influx is rapid enough to imply a close association of $K_{(Ca)}$ channels with Ca^{2+} channels (Roberts *et al.*, 1990; Roberts *et al.*, 1991). Given the importance of Ca^{2+} channels in the activation of $K_{(Ca)}$ channels, alterations in the interaction between these channel types may occur during development, possibly modifying the way in which the adrenal medulla responds to hypoxia.

The following set of experiments aimed firstly to find if there are differences between adult and fetal sheep AMCCs in the relative proportion of the various subtypes of VGCCs present and the contribution each of these subtypes makes to Ca^{2+} entry during a sustained membrane depolarisation, as these factors may underlie developmental differences in cell function. As $K_{(Ca)}$ currents constitute a large proportion of the K^+ current in sheep AMCCs and can modulate the electrical

excitability of these cells, the contribution each Ca^{2+} channel type makes to the activation of $K_{(Ca)}$ current was also studied. This revealed whether a specific Ca^{2+} channel type contributes preferentially to $K_{(Ca)}$ channel activation and provide evidence of Ca^{2+} and K^+ channel colocalisation in AMCCs.

5.2 Methods

5.2.1 Isolation of adrenal chromaffin cells

Merino ewes between 95 and 145 days gestation (term is 147 ± 3 days) were used for these experiments and fetal and adult AMCCs were enzymatically prepared as previously described in Section 2.2.1.

5.2.2 Electrophysiology

Whole-cell recordings were conducted according to the methods previously described in Section 2.2.2. The only variation from this was that when Ca^{2+} currents were recorded, cells were bathed in a solution containing (mM): $CaCl_2$, 60; $MgCl_2$, 2; NaCl, 70; TEA-Cl, 70; HEPES, 10; NPPB, 0.1; TTX, 3×10^{-4}. The Ca^{2+} channel antagonists nifedipine, ω-conotoxin, ω-agatoxin TK and the voltage-gated Ca^{2+} channel blockers, cadmium (Cd^{2+}) and cobalt (Co^{2+}), were applied through the perfusion solution for 3 to 5 minutes.

When looking at the effect these Ca^{2+} channel antagonists have on either Ca^{2+} or K^+ currents, the area under the current density-voltage curve was used (GraphPad Prism 3.0, GraphPad Software Inc., San Diego, USA). Area under the curve provides a single measure for an effect across a range of voltages.

5.2.3 Calcium Imaging

Imaging of intracellular Ca^{2+} levels occurred as outlined in Section 2.2.3. Cells were depolarised by superfusing them with a high $[K^+]$ external solution (containing (in mM) NaCl, 94; KCl, 50; $CaCl_2$, 2; $MgCl_2$, 2; HEPES, 10; glucose, 10; with pH adjusted to 7.4 with NaOH) with or without a Ca^{2+} channel antagonist. The ensuing increase in mean pixel value of the cell was then measured 60 seconds after the application of high $[K^+]$ solution, where the pixel values were a grey scale ranging from 0 to 255. This ratio was calculated according to the equation:

$$R = (F-F_{min}) / F_{min}$$

where: R = ratio of Ca^{2+} fluorescence change

F_{min} = mean fluorescence intensity level during control period

F = mean fluorescence intensity level after 1 min in high $[K^+]$ solution

The R value with and without the application of specific Ca^{2+} channel blockers was calculated and the former divided by the latter. This value was subtracted from one and multiplied by 100 to represent the contribution each Ca^{2+} channel makes to Ca^{2+} entry as a percentage, as shown below:

$$\% \text{ contribution of } Ca^{2+} \text{ channel} = (1 - (R_{with} / R_{without})) \times 100$$

where: R_{with} = ratio of fluorescence change with a Ca^{2+} channel blocker present

$R_{without}$ = ratio of fluorescence change without a Ca^{2+} channel blocker present

5.2.4 Drugs

The Ca^{2+} channel antagonists nifedipine (L-type blocker), ω-conotoxin GVIA (N-type), ω-agatoxin TK (P/Q-type) and the non-specific VGCC blockers cadmium chloride and cobalt chloride were obtained from Sigma Chemicals, USA. In all experiments these antagonists were used at the concentrations of 1 μM, 100 nM, 200 nM, 200 μM and 5 mM, respectively. Fluo-3 AM and Pluronic F-127 were obtained from Molecular Probes, USA.

5.2.5 Statistics

Results are expressed as means ± SEM. The effect of individual treatments, such as specific pharmacological blockade of Ca^{2+} channels, were tested by the Student's paired t-tests comparing responses with and without the Ca^{2+} channel antagonist. The difference in areas under the curve between adult and fetal groups was tested using unpaired t-tests. $p<0.05$ is taken as the minimum level of significance.

5.3 Results

5.3.1 Ontogenic differences in the contribution of Ca^{2+} channels to Ca^{2+} influx

Both 140 day fetal (135-145 days gestation) and adult ovine AMCCs were used for these experiments where the relative contribution of Ca^{2+} channels to changes in $[Ca^{2+}]_i$ was the focus, with particular attention being made to any ontogenic differences which may exist. Both fetal and adult AMCCs have VGCCs. Exposure to high $[K^+]$ increased $[Ca^{2+}]_i$ which was reduced in each case by 96% when Ca^{2+} was absent from the extracellular solution (Fig. 5.1), indicating that Ca^{2+} entering the cytosol in these experiments is derived from extracellular sources. In fetal cells, Cd^{2+} caused an 85 ± 4% decrease in Ca^{2+} entry evoked by depolarisation with high $[K^+]$ solution, while in adult cells this value was 97 ± 4%.

Specific Ca^{2+} channel antagonists were employed to investigate the contribution of L-, N- and P/Q-type Ca^{2+} channels to the changes in $[Ca^{2+}]_i$ in fetal and adult cells exposed to the high $[K^+]$ solution. The L-type Ca^{2+} channel antagonist, nifedipine, caused a decrease in cell fluorescence of 18 ± 10% in fetal and 18 ± 12% in adult cells exposed to high extracellular $[K^+]$. The N-type Ca^{2+} channel blocker, ω-Conotoxin GVIA, produced a 48 ± 10% and 48 ± 11% decrease in Ca^{2+} influx in fetal and adult cells, respectively. Finally, the specific P/Q-type antagonist, ω-agatoxin TK, produced a decrease in cell fluorescence of 41 ± 8% in fetal cells and 38 ± 9% in adult cells stimulated with the high $[K^+]$ solution.

5.3.2 Ontogeny of Ca^{2+} channel contribution to total Ca^{2+} current

In the following group of experiments involving electrophysiological analysis of Ca^{2+} currents present in AMCCs, both 100 day fetal (90-110 days gestation) and adult ovine AMCCs were utilised. In order to measure only Ca^{2+} currents, blockers of Cl^-, Na^+ and K^+ channels were included in the extracellular and intracellular solutions. Current density was plotted against voltage to decrease the variability in results for separate cells.

The inward Ca^{2+} current peaked at around 30 mV when stepped from a holding potential of -80 mV, a peak which is at a more positive voltage than previously seen in these cells (usually around 5 mV, see Figure 2.1B). This is attributed to the high concentration of Ca^{2+} ions (60 mM) in the extracellular solution causing a surface charge effect which shifts the current-voltage relationship to the right. The reason for using such a high external Ca^{2+} concentration was to aid in identifying any presence of T-type Ca^{2+} channels. Currents resulting from these channels are characterized as being small in their maximum amplitude compared to that of high threshold activated currents, fast-inactivating, activating at around -50 mV and having a peak current at -10 mV (Bournaud *et al.*, 2001). Ca^{2+} currents corresponding to these biophysical properties were identified (Fig 5.2) in 13 of 26 fetal cells and 16 of 22 adult cells tested in these experiments verifying the existence of T-type Ca^{2+} currents in both fetal and adult sheep AMCCs.

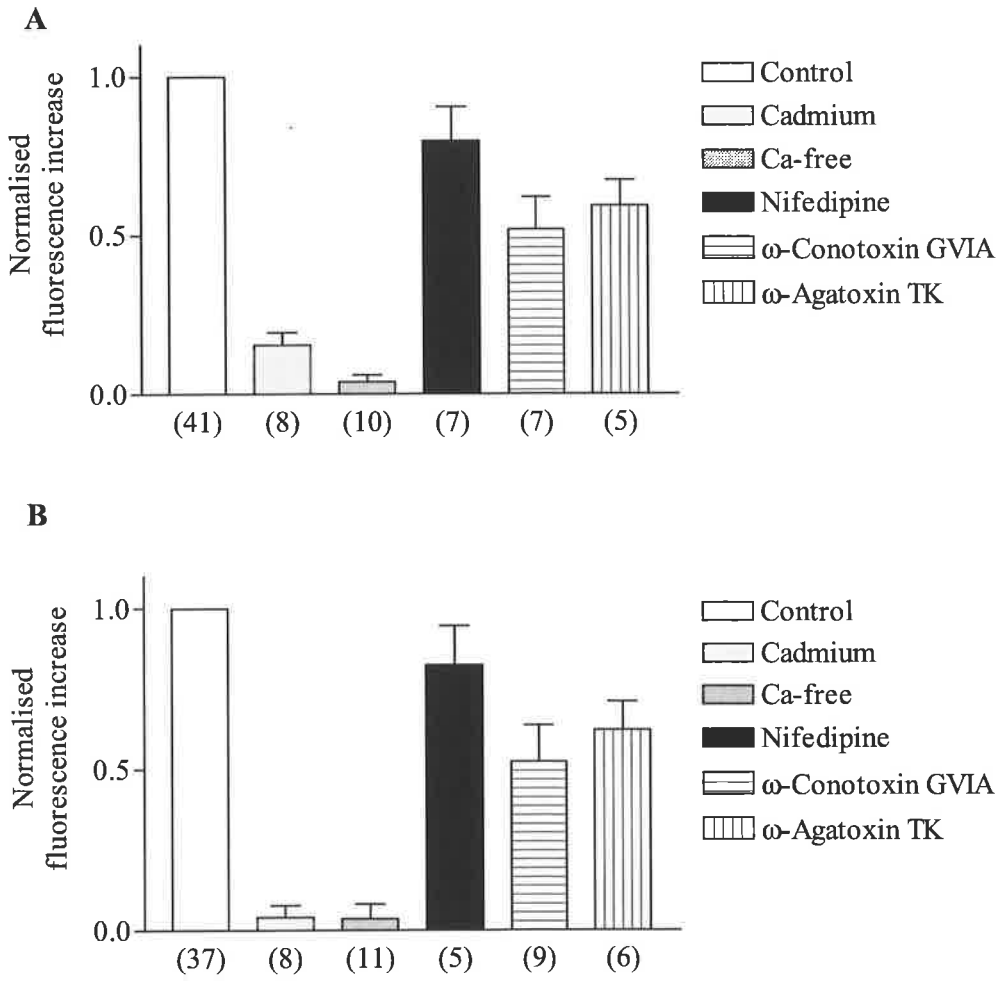


Figure 5.1: Contribution of VGCC subtypes to Ca^{2+} influx in AMCCs isolated from fetal (A) and adult (B) sheep. Fluorescence of fluo-3 in each cell, measured one minute after the application of high $[K^+]$ solution, was compared to fluorescence in the same solution but with a specific Ca^{2+} channel antagonist added or with no Ca^{2+} in the external solution. All treatments significantly reduced ($p<0.05$) the fluorescence below that measured in extracellular solution containing high $[K^+]$ without any channel blockers, the value of which has been normalised to 1. Number of cells in each data set is in parentheses below each column.

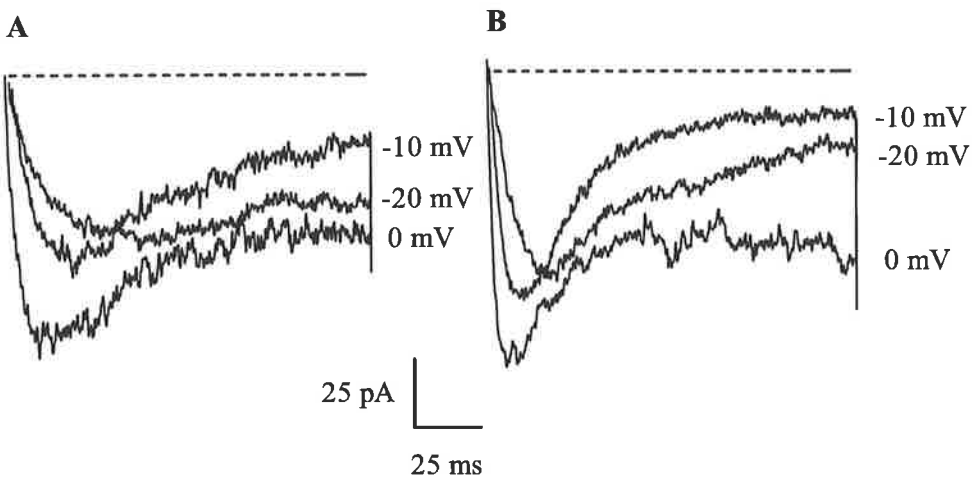


Figure 5.2: Current traces illustrating transient T-type Ca^{2+} currents in fetal (A) and adult (B) sheep. Cells were held at -80 mV and stepped to 0, -10 and -20 mV for 100 ms.

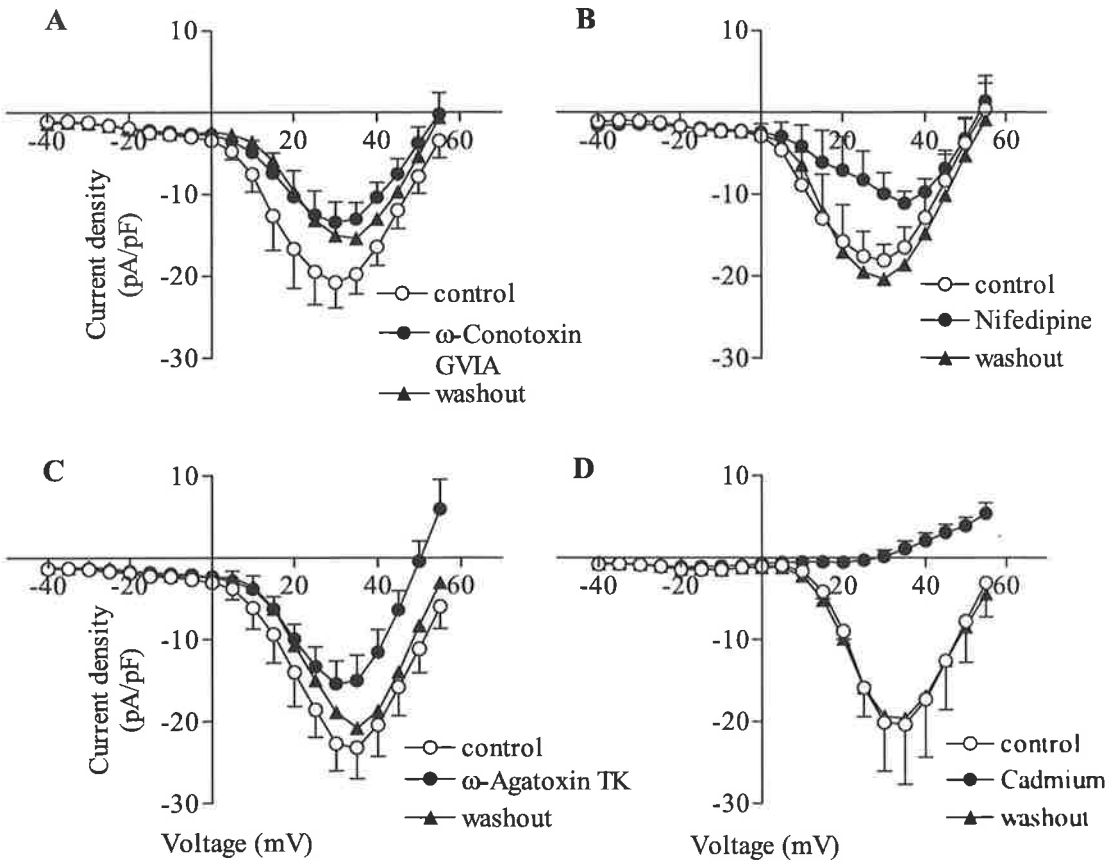


Figure 5.3: Contribution of specific Ca^{2+} channels to whole cell Ca^{2+} current in fetal AMCCs. The current density-voltage relationship of Ca^{2+} current is plotted with the Ca^{2+} channel blocked in each panel being **A** N-type (n=6), **B** L-type (n= 6), **C** P/Q-type (n= 5) and **D** all (n=4). Cells were held at -80 mV and stepped to potentials ranging from -40 to +55 mV for 100 ms in increments of 5 mV.

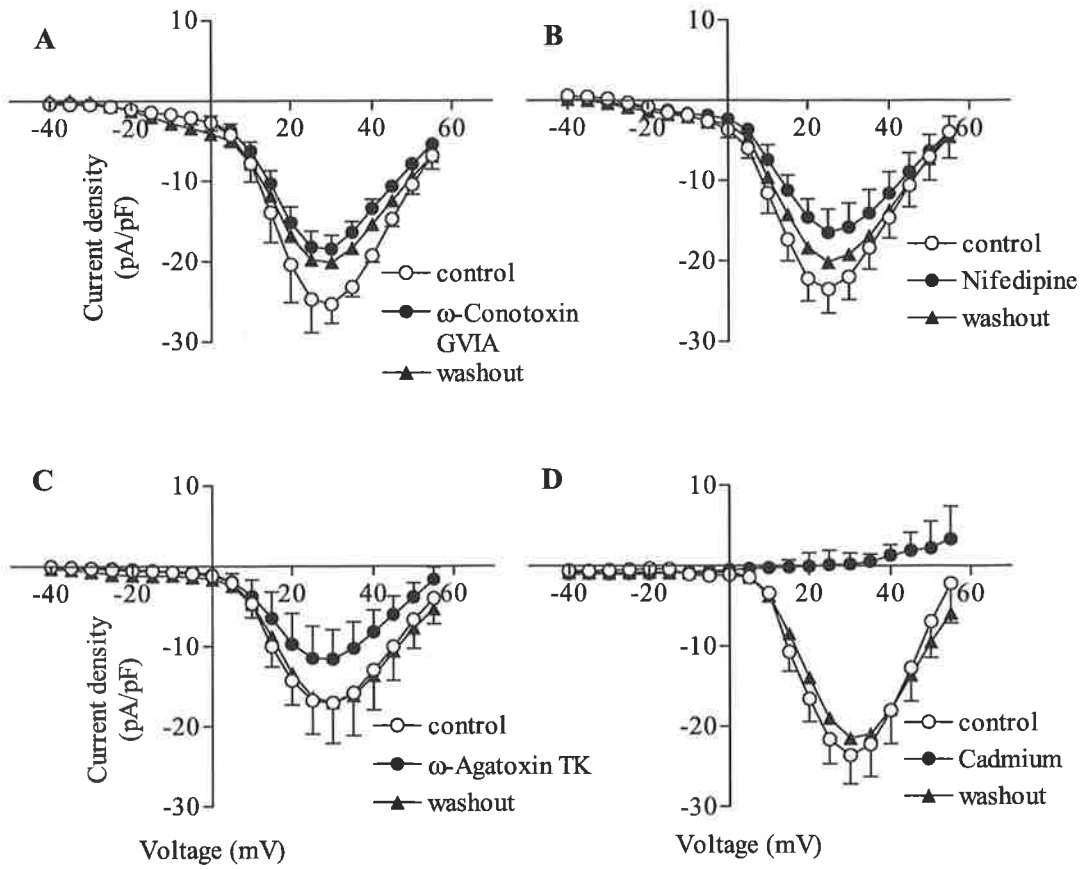


Figure 5.4: Contribution of specific Ca^{2+} channels to whole cell Ca^{2+} current in adult AMCCs. The current density-voltage relationship of Ca^{2+} current is plotted with the Ca^{2+} channel blocked in each panel being **A** N-type (n=4), **B** L-type (n= 8), **C** P/Q-type (n= 5) and **D** all (n=5). Cells were held at -80 mV and stepped to potentials ranging from -40 to +55 mV for 100 ms in increments of 5 mV.

The amount of Ca^{2+} current attributed to each Ca^{2+} channel subtype was determined by measuring the area under the current density-voltage curve with and without specific Ca^{2+} channel antagonists. This allows the contribution of each Ca^{2+} channel type to the total Ca^{2+} current to be evaluated across a full range of voltages instead of at a single voltage. Given that the voltage dependence of different Ca^{2+} channels varies, looking at the effect of Ca^{2+} channel antagonists at a single voltage may give an inaccurate profile of the full contribution each Ca^{2+} channel makes to the total Ca^{2+} current.

The N-type Ca^{2+} channel antagonist, ω-conotoxin GVIA, caused a decrease in inward Ca^{2+} current (Fig. 5.3A; $p<0.01$) of 33 ± 12% in fetal cells and of 25 ± 7% in adult AMCCs (Fig. 5.4A; $p<0.01$). Application of nifedipine, the specific L-type Ca^{2+} channel antagonist, resulted in a 32 ± 10% and 31 ± 17% decrease in inward Ca^{2+} current in fetal (Fig. 5.3B; $p<0.01$) and adult (Fig. 5.4B; $p<0.001$) AMCCs, respectively. The P/Q-type channel blocker, ω-agatoxin TK, significantly reduced Ca^{2+} current in fetal cells by 35 ± 7% (Fig. 5.3C; $p<0.01$) and in adult cells by 32 ± 22% (Fig. 5.4C; $p<0.01$). A non-selective blocker of VGCCs, Cd^{2+}, abolished the inward Ca^{2+} current in both fetal (Fig. 5.3D; $p<0.01$) and adult cells (Fig. 5.4D; $p<0.01$). From these results it appears that little developmental change occurs in the Ca^{2+} channels present in ovine AMCCs and that L-, N- and P/Q-type channels contribute equally to the total Ca^{2+} current in these cells. One difference which is evident from the Ca^{2+} current results is that, in fetal cells, an outward current is activated at positive potentials upon blockade with ω-agatoxin TK. The identity of this outward current is unclear, as is the reason why it is evident upon application of ω-agatoxin TK.

5.3.3 Contribution of Ca^{2+} channel subtypes to $K_{(Ca)}$ current activation

As with Ca^{2+} currents, in order to determine the effect of Ca^{2+} channel blockade on K^+ current at several potentials, the decrease in the area under the curve was measured. In fetal cells the simultaneous blockade of L-, N- and P/Q-type channels reduced K^+ current density by 72 ± 2% (Fig. 5.5D; $p<0.05$). A similar result was seen in adult cells with the application of the non-specific Ca^{2+} channel blocker, Co^{2+}, decreasing K^+ current density by 78 ± 7% (Fig 5.6D; $p<0.01$). These results indicate that of the total outward K^+ current in these cells, approximately 25% is not Ca^{2+}-dependent.

N-type Ca^{2+} channel blockade caused a similar reduction in K^+ current in both fetal and adult cells of 32 ± 10% (Fig. 5.5A; $p<0.01$) and 30 ± 13% (Fig. 5.6A; $p<0.05$), respectively. L-type Ca^{2+} channel blockade reduced K^+ current by 35 ± 11% (Fig. 5.5B; $p<0.02$) in fetal and 30 ± 14% (Fig. 5.6B; $p<0.01$) in adult cells, while in fetal cells the blockade of both N- and L-type Ca^{2+} channels decreased the K^+ current by 62 ± 12% (Fig. 5.5D; $p<0.05$). The P/Q-type antagonist, ω-agatoxin TK, had the greatest effect of all three Ca^{2+} channel blockers by decreasing K^+ current in fetal cells by 47 ± 10% (Fig. 5.5C; $p<0.05$) and in adult cells by 49 ± 9% (Fig. 5.6C; $p<0.01$).

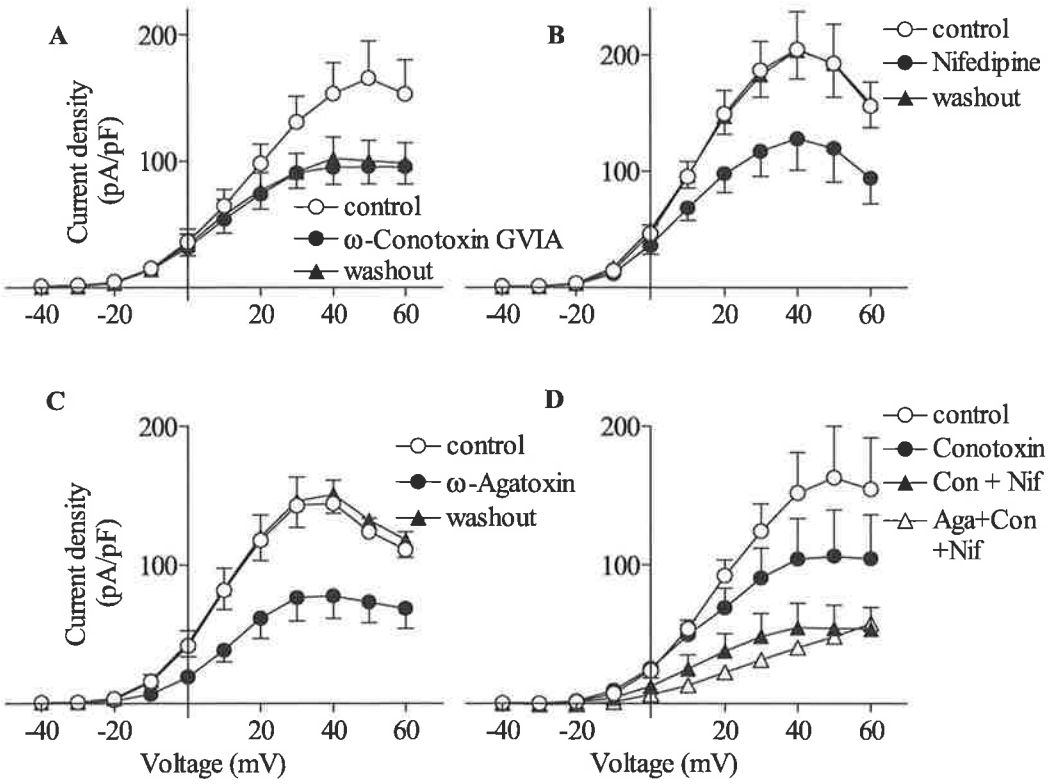


Figure 5.5: Contribution of Ca^{2+} entry through specific Ca^{2+} channels to the activation of whole cell $K_{(Ca)}$ current in fetal AMCCs. The current density-voltage relationship of $K_{(Ca)}$ current is plotted and the Ca^{2+} channel blocked in each panel is **A** N-type (n=7), **B** L-type (n= 7), **C** P/Q-type (n= 5) and **D** N-, L- and P/Q-type (n=3). Cells were held at -80 mV and stepped to potentials ranging from -40 to +60 mV for 100 ms in increments of 10 mV.

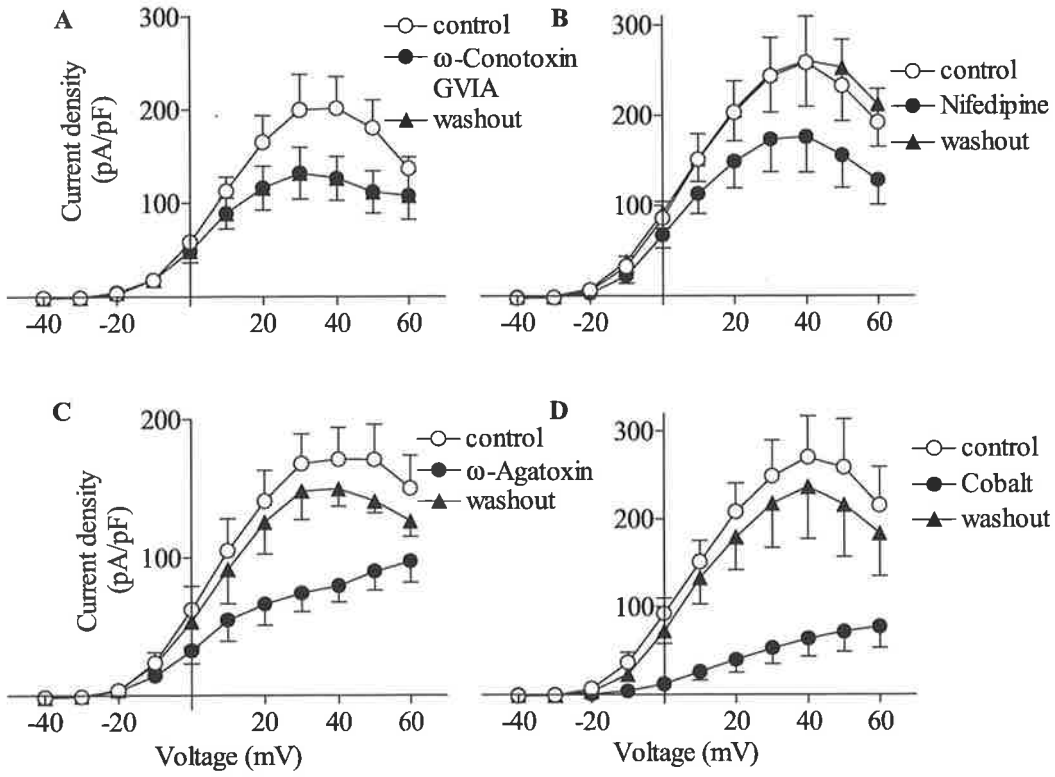


Figure 5.6: Contribution of Ca^{2+} entry through specific Ca^{2+} channels to the activation of whole cell $K_{(Ca)}$ current in adult AMCCs. The current density-voltage relationship of $K_{(Ca)}$ current is plotted and the Ca^{2+} channel blocked in each panel is **A** N-type (n=4), **B** L-type (n= 6), **C** P/Q-type (n= 4) and **D** all (n=6). Cells were held at -80 mV and stepped to potentials ranging from -40 to +60 mV for 100 ms in increments of 10 mV.

5.4 Discussion

In this investigation, the ontogeny of Ca^{2+} channels in fetal and adult AMCCs was analysed. This was undertaken in order to find whether functional differences between these cells obtained at different developmental stages is due to changes in the proportion of various Ca^{2+} channel types and the contribution they make in activating $K_{(Ca)}$ current.

By using Ca^{2+} imaging techniques in single cells the contribution that different Ca^{2+} channels make to Ca^{2+} influx upon membrane depolarisation was examined. Fetal cells differed from adult AMCCs in having a component of voltage-gated Ca^{2+} entry which is not blocked by cadmium. One possibility for how Ca^{2+} entry occurs in the presence of Cd^{2+} is through Ca^{2+}-permeable amyloid β-peptide channels. Prolonged hypoxia induces the formation of amyloid β-peptide channels in PC-12 cells (Taylor *et al.*, 1999). As amyloid β-peptide channels are not blocked by Cd^{2+}, the increased presence of these channels in chronically hypoxic PC-12 cells underlies the increased level of Cd^{2+}-reseistant Ca^{2+} entry seen during membrane depolarisation with high extracellular K^+ (Taylor *et al.*, 1999). Whether these channels are present in fetal AMCCs remains unconfirmed.

In both fetal and adult AMCCs, L-type Ca^{2+} channels contribute only a small amount to the overall Ca^{2+} entry, with N- and P/Q-type Ca^{2+} channels being responsible for approximately 80% of Ca^{2+} entry. The different contribution of the various subtypes of VGCCs to Ca^{2+} entry when AMCCs are treated with high K^+ concentrations contrasts to the electrophysiological measurements of Ca^{2+} currents, where L-, N- and P/Q-type Ca^{2+} channels each contributed about one third of the total Ca^{2+} current.

This difference is most likely due to the fact that the Ca^{2+} imaging data was obtained by inducing Ca^{2+} entry with an increased external concentration of K^+ ions (50 mM). Knowing the external K^+, Na^+, and Cl^- concentrations and using estimates of the

internal concentration of these ions, the Goldman-Hodgkin-Katz voltage equation predicts that the membrane will be depolarised to a potential of around -30 mV by the application of 50 mM K^+ in the extracellular solution. The voltage at which recombinantly expressed HVA Ca^{2+} channels begin to be activated is approximately -30 mV for L-type (Allen *et al.*, 1998), -40 mV for N-type (Wakamori *et al.*, 1998) and -40 mV for P/Q-type (Allen *et al.*, 1998) Ca^{2+} channels, and -40 mV and -50 mV for native Ca^{2+} channels in either AMCCs (Gandia *et al.*, 1993) or supraoptic neurones (Joux *et al.*, 2001), respectively. Therefore, the level of depolarisation produced by this high $[K^+]$ solution will only partially activate the opening of the HVA Ca^{2+} channels, making it difficult to compare the contribution these channels make towards total Ca^{2+} current across a full range of activating potentials.

In addition to the varying degree of activation occurring in these Ca^{2+} imaging experiments, there is the additional issue of Ca^{2+} channel inactivation to consider. $[Ca^{2+}]_i$ was routinely recorded after one minute of depolarisation, long enough for Ca^{2+}-dependent inactivation of all three Ca^{2+} channel types to take effect. The pore-forming α subunit of L-, N- and P/Q-type Ca^{2+} channels have been recently identified in bovine AMCCs (Wick *et al.*, 1996; Garcia-Palomero *et al.*, 2000) and results from this current study clearly indicate that these channels are functional in the sheep adrenal medulla also. Recombinantly expressed L-type channels are inactivated by Ca^{2+} (Pitt *et al.*, 2001) within approximately 400 ms (Zuhlke *et al.*, 2000) while recombinant P/Q-type channels have a much slower rate of Ca^{2+}-dependent inactivation that takes full effect after 2000 ms (DeMaria *et al.*, 2001). The expressed $\alpha_1$2.2 subunit (N-type Ca^{2+} channel α subunit) is inactivated by Ca^{2+} at a rate which appears intermediate to L- and P/Q-type Ca^{2+} channels Wakamori *et al.*, 1998). The rate at which this Ca^{2+}-dependent inactivation of Ca^{2+} channels occurs is markedly altered, however, depending on the β-subunit with which they

are co-expressed (Cens *et al.*, 1999; Tsunemi *et al.*, 2002) and when α and β subunits are coexpressed, the kinetics of activation more closely resemble the kinetics of native channels (Birnbaumer *et al.*, 1998). This is illustrated in a high percentage of bovine AMCCs where N-type Ca^{2+} channels do not inactivate during long depolarisations (Artalejo *et al.*, 1992) due to coexpression of the $\alpha_1 2.2$, β_{2a} and α_2/δ subunits in these cells (Cahill *et al.*, 2000).

It seems, therefore, that the time course of Ca^{2+}-dependent inactivation of these channels is reflected in the level that each contributes to Ca^{2+} entry during a sustained membrane depolarisation. A fast rate of channel inactivation would explain why blockade of L-type channels has such little effect on Ca^{2+} entry compared to the blockade of N- and P/Q-type channels, which are open longer and allow more Ca^{2+} to enter through them. Although these Ca^{2+} imaging results do not represent how Ca^{2+} enters AMCCs during a strong depolarisation which fully activates all Ca^{2+} channels, they do illustrate that ontogenic differences between adult and fetal AMCCs do not exist with regards to the proportion each Ca^{2+} channel subtype contributes to Ca^{2+} entry during a moderate membrane depolarisation.

The Ca^{2+} current traces recorded from adult and fetal AMCCs also display a component which is characteristic T-type Ca^{2+} currents. T-type currents are transient, fast-inactivating, have a small single channel conductance and a resultant small current amplitude (Tsien *et al.*, 1988). These features make this current type particularly difficult to detect which was why such a high external Ca^{2+} concentration was chosen for the recording solution. Antagonists of T-type channels such as amiloride (Williams *et al.*, 1999) and mibefradil (Luscher *et al.*, 1997) lack specificity and partially block other Ca^{2+} channels (Hollins and Ikeda, 1996; Luscher *et al.*, 1997) so the use of these agents seemed inappropriate in these experiments. T-type Ca^{2+} currents have been detected previously by

only three groups and in all cases these currents appeared only in a subset of adult rat (23% of cells tested) and bovine (70% of cells tested) AMCCs (Diverse-Pierluissi *et al.*, 1991; Hollins and Ikeda, 1996) and a subset of fetal rat AMCCs (54% of cells tested) (Bournaud *et al.*, 2001) making this current type far more difficult to detect than HVA Ca^{2+} currents. Using this high Ca^{2+} concentration in the external solution for these current experiments, T-type Ca^{2+} currents were detected in a subset of both fetal and adult sheep AMCCs.

In ovine AMCCs, L-, N- and P/Q-type Ca^{2+} channels contribute approximately one third each to the total Ca^{2+} current, with no difference in the ontogeny of Ca^{2+} channels between fetal and adult cells. While there may appear to be a slight decrease in N-type Ca^{2+} channel levels in adult cells, this difference is not statistically significant and is likely due to natural variations in channel expression which occur between cells. There are large species differences in the contribution made by L-, N- and P/Q-type Ca^{2+} channels to the total Ca^{2+} current, as the respective contribution of these channels is 20%, 20% and 60% in human (Albillos *et al.*, 1996b), 50%, 30% and 20% in rat (Gandia *et al.*, 1995) and 15%, 80% and 5% in pig (Kitamura *et al.*, 1997) AMCCs.

While all three Ca^{2+} channel types contribute equally to Ca^{2+} entry in sheep AMCCs, it is not known whether any specific Ca^{2+} channel is involved in $K_{(Ca)}$ channel activation or whether Ca^{2+} and $K_{(Ca)}$ channel colocalisation changes during development. In rat AMCCs, some BK channels are exposed to $[Ca^{2+}]_i$ of at least 60 μM, however, this $[Ca^{2+}]_i$ increase is highly nonuniform, resulting in only 10-20% of BK channels being exposed to sufficient $[Ca^{2+}]_i$ for them to be activated during a normal action potential (Prakriya *et al.*, 1996). This raises the issue of whether the Ca^{2+} which regulates BK channel opening enters the cell via a particular type of Ca^{2+} channel. Further studies on rat AMCCs reveal that the activation of BK channels occurs mainly via Ca^{2+} entry through L- and Q-type Ca^{2+} channels, even though N- and P-type currents are also present (Prakriya

and Lingle, 1999). The coupling of these Ca^{2+} channels with BK channels is tight, with a distance from each other on the cell membrane of 50 to 160 nm and Ca^{2+} influx through multiple Ca^{2+} channels appears to regulate any individual BK channel (Prakriya and Lingle, 2000). Functional colocalisation of Ca^{2+} and $K_{(Ca)}$ channels has also been reported in the frog neuromuscular junction where BK channels are clustered together with Ca^{2+} channels at the presynaptic membrane facing the postsynaptic membrane, allowing for fast activation of BK channels upon Ca^{2+} entry into the terminal (Robitaille *et al.*, 1993).

Both SK and BK channels are present in fetal and adult sheep AMCCs and the degree and manner in which these channels are activated *in vivo* by external stimuli changes upon the development of adrenal gland innervation. Therefore, the possibility that the activation of $K_{(Ca)}$ channels occurs through different types of Ca^{2+} channels in fetal and adult AMCCs was investigated to establish whether this underlies developmental differences in $K_{(Ca)}$ channel function.

Patch clamp results indicate that approximately 75% of the total K^+ current is Ca^{2+}-dependent, as indicated by the effect of Co^{2+} or simultaneous application of nifedipine, ω-conotoxin GVIA and ω-agatoxin TK. The effect of blocking L-, N- or P/Q-type Ca^{2+} channels separately however, results in around 30%, 30% and 50% reduction in whole cell K^+ current, representing approximately 40%, 40% and 67% of the total $K_{(Ca)}$ current, respectively. Also, in fetal cells, blockade of both N- and L-type Ca^{2+} channels reduces $K_{(Ca)}$ current by around 83%, leaving only 17% of $K_{(Ca)}$ current which P/Q-type channels would supposedly be responsible for activating. These results indicate that there is a high degree of overlap in the mechanism by which L-, N- and P/Q-type Ca^{2+} channels activate $K_{(Ca)}$ currents in adult and fetal sheep AMCCs.

While L-, N- and P/Q-type Ca^{2+} channels all contribute equally to total Ca^{2+} current, blockade of P/Q-type currents causes the largest reduction in $K_{(Ca)}$ currents. There

is no indication from the Ca^{2+} imaging data that Ca^{2+} entry through P/Q-type channels is larger than that through N-type channels. Taken together, it appears that P/Q-, more than N-, type Ca^{2+} channels are preferentially colocalised with $K_{(Ca)}$ channels.

In considering the present results on the contribution which different Ca^{2+} channel subtypes make toward Ca^{2+} entry and $K_{(Ca)}$ channel activation, one must remember that they are obtained from cultured cells and that changes in channel expression can occur in culture. Approximately 34% of Ca^{2+} current in mouse adrenal slices has been attributed to R-type channels (Albillos *et al.*, 2000), as opposed to cultured mouse AMCCs which contain no current due to R-type channels (Hernandez-Guijo *et al.*, 1998b). This difference within the same species is an example of changes in channel type proportions which may occur due to time in culture and hence, while we find that L-, N- and P/Q-type channels constitute all the Ca^{2+} currents in adult and fetal cultured cells, this may not necessarily be the case *in vivo*.

From this study, several conclusions can be made on the ontogeny of Ca^{2+} channels and their contribution to Ca^{2+} entry and $K_{(Ca)}$ channel activation. No developmental differences were observed between fetal and adult AMCCs as L-, N- and P/Q-type Ca^{2+} channels all contribute equally to the Ca^{2+} current in fetal and adult cells. N- and P/Q-type Ca^{2+} channels, rather than L-type Ca^{2+} channels, are principally responsible for Ca^{2+} entry during a sustained membrane depolarisation in both adult and fetal AMCCs, most likely due to differences in the rate of Ca^{2+}-dependent inactivation of these Ca^{2+} channels. The high proportion of $K_{(Ca)}$ current activation by P/Q-type channels in adult and fetal AMCCs may signify a tighter coupling of this Ca^{2+} channel to $K_{(Ca)}$ channels. While it is not important to the aims of the present study, further insight into the biology of ovine AMCCs might be obtained by using specific $K_{(Ca)}$ channel antagonists to investigate whether these P/Q-type Ca^{2+} channels are preferentially colocalised with either SK or BK channels.

6. GENERAL DISCUSSION

The major aims of this project were to find the mechanisms, at the cellular level, which underlie the direct, non-neurogenic, response to hypoxia in sheep AMCCs. This involved identifying the K^+ currents suppressed by hypoxia and the intracellular pathways by which this reduction in environmental P_{O_2} levels is transduced into the physical alteration of the channel protein. The underlying mechanism of how the direct hypoxic response is suppressed upon the development of adrenal gland innervation was also investigated. This involved examining the effect that opioid peptides released from splanchnic nerve terminals have on AMCC ion channels. The Ca^{2+} and K^+ channels present in fetal and adult AMCCs and how these channels interact with each other, was also investigated, as differences in these factors may also underlie the different response to hypoxia in adult and fetal AMCCs.

In both adult and fetal AMCCs, hypoxia reduces the K^+ current amplitude significantly by inhibiting SK channels. In fetal AMCCs BK current amplitude is also depressed by hypoxia. The hypoxic suppression of these $K_{(Ca)}$ currents is not due to any hypoxia-induced decrease in Ca^{2+} current amplitude, indicating that hypoxia acts only on $K_{(Ca)}$ channels. The closure of SK channels during hypoxia appears to have functional implications, as the specific blockade of these channels with either apamin or curare causes a shift in the membrane reversal potential similar to that caused by hypoxia. This depolarisation causes the opening of voltage-gated Ca^{2+} channels and allows Ca^{2+} from the extracellular fluid to enter the cell, as demonstrated by the significant increase in $[Ca^{2+}]_i$ caused by either hypoxia, apamin or curare. The fact that $[Ca^{2+}]_i$ during hypoxia does not increase further when either of these two SK channel antagonists are simultaneously applied with hypoxia, indicates that SK channels mediate the membrane depolarisation during hypoxia.

While BK current is also depressed by hypoxia, the closure of these channels does not alter the resting membrane potential, as indicated by the lack of effect TEA has on the reversal potential and $[Ca^{2+}]_i$. The application of TEA and apamin together increases the Ca^{2+} entry caused by apamin alone in fetal AMCCs, illustrating that closure of these BK channels during hypoxia may have a functional role in augmenting the membrane depolarisation and increase in $[Ca^{2+}]_i$ initiated by SK channel closure.

The identity of the intracellular system which is responsible for sensing changes in local P_{O_2} levels and transducing these changes to the effector K^+ channels shows cell type- and species-specific diversity across a broad range of O_2-sensitive tissues. Intracellular ROS levels appear to modulate the opening of K^+ channels in adult AMCCs, and this is in agreement with the theory that the intracellular redox state regulates O_2-sensitive ion channel function. Of the two ROS producing systems investigated in this study, mitochondria, and not NADPH oxidase, appear to be involved in the O_2 sensing mechanism of AMCCs possibly due to a shift to a more reduced redox state within the cytosol during hypoxia caused by a decrease in ROS production. The identity of the ion channel(s) effected by these alterations in redox state appears to be the O_2-sensitive SK and BK channels, as an inhibition of ROS production caused by rotenone, results in a loss of hypoxic suppression of K^+ current.

It is well established that innervation of the adrenal gland results in the suppression of the direct response of AMCCs to hypoxia. This is not due to the actions of the cholinergic agonist acetylcholine being released from innervating fibres onto the AMCCs, but seems likely to involve the release of some other factor. Endogenous opioid peptide release from splanchnic nerve terminals appears a likely mechanism for this suppression of the direct hypoxic response. The application of μ- and κ-type opioid agonists to whole perfused fetal adrenal glands during hypoxia results in a significant reduction of hypoxia-

evoked catecholamine secretion. These agonists both significantly reduce hypoxia-induced Ca^{2+} influx, an effect which is in part due to their actions in reducing Ca^{2+} current amplitude, but mainly due to the increase in SK channel conductance by μ-type activation. This increase in SK channel conductance is likely to cause membrane hyperpolarisation, therefore offsetting any depolarisation caused by the hypoxic closure of SK channels. As closure of BK channels does not depolarise the membrane potential, this κ-type activation will have no effect on the resting membrane potential in these cells. The effects of these agonists on K^+ and Ca^{2+} currents could explain how endogenous opioid peptides might cause the suppression of the non-neurogenic hypoxic response upon adrenal gland innervation.

The possibility that the switch from non-neurogenic to neurogenic responses to hypoxia with the development of innervation may be due to changes in the proportion of Ca^{2+} channels present and their specific contributions to $K_{(Ca)}$ current activation, was investigated in fetal and adult sheep AMCCs. T-type Ca^{2+} currents were observed in a greater percentage of adult AMCCs than in fetal AMCCs. If T-type Ca^{2+} channels were present in fetal but not adult AMCCs, this may explain how hypoxia, which produces a membrane depolarisation which is relatively small compared to that caused by cholinergic stimulation, could evoke Ca^{2+} entry and catecholamine secretion in fetal but not adult cells. As this was not the case the contribution of other Ca^{2+} channel subtypes to total Ca^{2+} current and Ca^{2+} entry was also investigated. No significant differences between adult and fetal AMCCs in culture were observed with regards to the different types of Ca^{2+} channels present or the amount these channels contribute to the activation of Ca^{2+} and $K_{(Ca)}$ currents. The level that each Ca^{2+} channel is involved in Ca^{2+} entry during a sustained depolarisation and the effect that blockade of either SK or BK channels has on membrane reversal potential and Ca^{2+} entry is not developmentally different either. It seems, therefore, that

differences in the proportion of Ca^{2+} or $K_{(Ca)}$ channels present in fetal and adult AMCCs do not underlie different mechanisms they use to respond to hypoxia.

The results produced from this project provide some important topics which could be the focus of future investigations. One of these is the mechanism of O_2 sensitivity in AMCCs, a question which is currently being investigated in our laboratory. Using heterologously expressed SK channels, the sensitivity of SK1, SK2 and SK3 currents to hypoxia, as well as to reducing and oxidising agents, can be compared. If the redox state of the cell is found to underlie the effect of hypoxia, point mutations of single thiol-containing amino acids can be made in an attempt to discover the functional areas of these channels which are responsible for oxygen sensitivity.

It must also be remembered that the act of culturing cells from native tissue can effect the expression of ion channels as has been illustrated in a number of studies. It would be prudent, therefore, to look at the hypoxic response in AMCCs within adrenal gland slices, as this preparation more closely resembles *in vivo* conditions than does cell culture.

It would also be interesting to find whether the STREX splice variant of BK channels is present in slices. The fast-inactivating BK currents are no longer seen in our laboratory after AMCCs have been in culture for more than 24 hours and this may not occur when using slices. The addition of apamin to rat AMCCs which contain fast-inactivating BK currents produces tonic action potential firing and if cells in adrenal slices contain this type of BK channel, this may have functional implications during the hypoxic closure of SK channels.

The use of specific opioid receptor antagonists to study the effect endogenous opioid peptides have on hypoxia-induced catecholamine secretion in the fetus could provide *in utero* evidence that opioid receptor activation suppresses the direct hypoxic

response. Carrying this out *in utero* before and after adrenal innervation develops would enable direct correlations to be made between the effect of these antagonists on hypoxia-induced secretion and the development of adrenal gland innervation.

Adrenal medullary tumours, or pheochromocytoma, clinically manifest themselves in humans as hypertension, and this is due to uncontrolled release of catecholamines. The chromaffin cells of these tumors also have reduced or nonexistent responses to opioid peptides compared to normal AMCCs. It seems possible then that the release of catecholamines would normally be suppressed by opioid receptor activation, but is not occurring in these tumors due to some alteration in opioid receptor structure or the method in which these receptors interact with K^+ channels. Hence, investigations into the structure and function of opioid receptors in the adrenal medulla of people with hypertension could be a study of widespread significance.

While it currently seems that the intracellular level of ROS effects K^+ channel function, and that the mitochondria is the main system effecting these ROS levels, the actual components within the ETC which endow O_2 sensitivity are still not fully understood. The discovery of exactly how the adrenal medulla, and other O_2 sensing tissues, sense and transduce changes in P_{O_2} levels to create changes in ion channel function remains the last great unknown factor in this area, and one which may not be easily overcome.

APPENDIX 1 : μ- AND κ-TYPE OPIOID RECEPTOR AGONISTS SUPPRESS HYPOXIA-INDUCED CATECHOLAMINE SECRETION FROM WHOLE PERFUSED FETAL ADRENAL GLAND

The following data is included as support material for Chapter 4 which discusses the effect which opioid receptor agonists have on the hypoxic suppression of K^+ currents in adult AMCCs. The investigators who carried out this work were Michael Adams and Grigori Rychkov from the Adelaide University and Hans Holgert of the Karolinska Institute, Sweden. This appendix outlines the methods and results for a set of experiments involving catecholamine release measured from whole perfused fetal adrenal glands subjected to acute hypoxia with and without the presence of a μ- and κ-type opioid receptor agonist. The online analysis of catecholamine secretion using a carbon fibre electrode from the whole perfused adrenal gland that was used for this study is a novel approach. The wide dynamic range allows the detection of the small amounts of catecholamine secreted during hypoxic episodes as well as the large amounts released by stimulation of the nicotinic cholinergic receptors.

Methods

Animals

Merino cross ewes were used for these experiments which were approved by the Adelaide University Animal Ethics Committee. Ewes were killed with intravenous pentobarbitone (8.1 g), and adrenals were collected from five fetuses within the age group of 136 – 145 days gestation according to the methods previously described by Adams, Simonetta and McMillen (1996). Once collected, glands were placed in Ca^{2+}-free Locke's solution consisting of (mM): NaCl, 154; KCl, 5.6; $NaHCO_3$, 3.6; glucose, 5.6; HEPES, 5.0; pH 7.4, and kept on ice for a maximum of 15 minutes until gland dissection was carried out.

Adrenal gland perfusion

The perfusion of adrenal glands followed the method outlined previously by Adams, Simonetta and McMillen (1996). Adrenals were mounted in a perfusion chamber, which consisted of a glass funnel covered with a fine nylon mesh (2 x 2 mm) platform. Glands were perfused (1 ml/min) with Krebs solution containing (mM) NaCl, 140; KCl, 4; glucose, 10; $CaCl_2$, 2; $MgCl_2$, 2; HEPES, 10; pH 7.4, maintained at 37°C and continuously bubbled with O_2 (Po_2 = 587.2 ± 14.4 mmHg; n=5).

All adrenals were perfused for 90 minutes before being exposed to hypoxia. Adrenal hypoxia was generated by perfusion for 20 minutes with hypoxic Krebs solution (bubbled with 1% O_2 + 99% N_2; Po_2 = 38.5 ± 2.7 mmHg; n=5). The Po_2 of the perfusate was determined using an ABL 550 blood gas analyzer (Radiometer, Copenhagen, Denmark). The use of a three-way stopcock allowed for swift alternation between the normoxic and hypoxic Krebs buffers.

Catecholamine measurement using online analysis

A pre-cut 10 µm diameter carbon fibre electrode (World Precision Instruments, USA), was positioned within the adrenal gland effluent. A holding voltage of +700 mV was applied between the carbon fibre tip and the reference electrode to allow oxidation of released catecholamines. Amperometric responses were monitored with an List EPC-7 amplifier (List, Darmstadt, Germany), collected at 4 kHz, digitised with a TL-1 DMA acquisition system (Axon Instruments) and monitored online with the Fetchex software from the pCLAMP 6.0 suite (Axon Instruments).

Statistics

Statistical analysis of results from the whole perfused fetal adrenal gland experiments was carried out so that the adrenal catecholamine responses to hypoxia in each experiment were expressed in relation to the mean level of catecholamine secretion in the 12 min baseline period preceding the onset of hypoxia. The catecholamine responses to hypoxia in the presence or absence of the opioid agonist were then compared using multifactorial ANOVA with experimental treatment (hypoxia alone or hypoxia + opioid) and sampling time as the specified variables. The Duncan's New Multiple Range Test was used post-ANOVA to identify significant differences between mean values. A probability level of 5% ($p<0.05$) was taken as significant for all analyses.

Results - Perfused fetal adrenal gland

Opioid agonists and hypoxia-evoked catecholamine secretion

Allowing the effluent of the perfused adrenal gland to flow over the carbon fibre electrode enabled on-line measurement of catecholamine secretion to take place. A calibration curve was constructed by adding bolus amounts of adrenaline to the solution perfusing the carbon fibre over a range of concentrations (Appendix Fig. 1 *Inset*). The linear relationship between the current amplitude recorded and rising catecholamine levels demonstrates that the data obtained using the on-line method is directly related to levels of catecholamines secreted from the perfused adrenal glands.

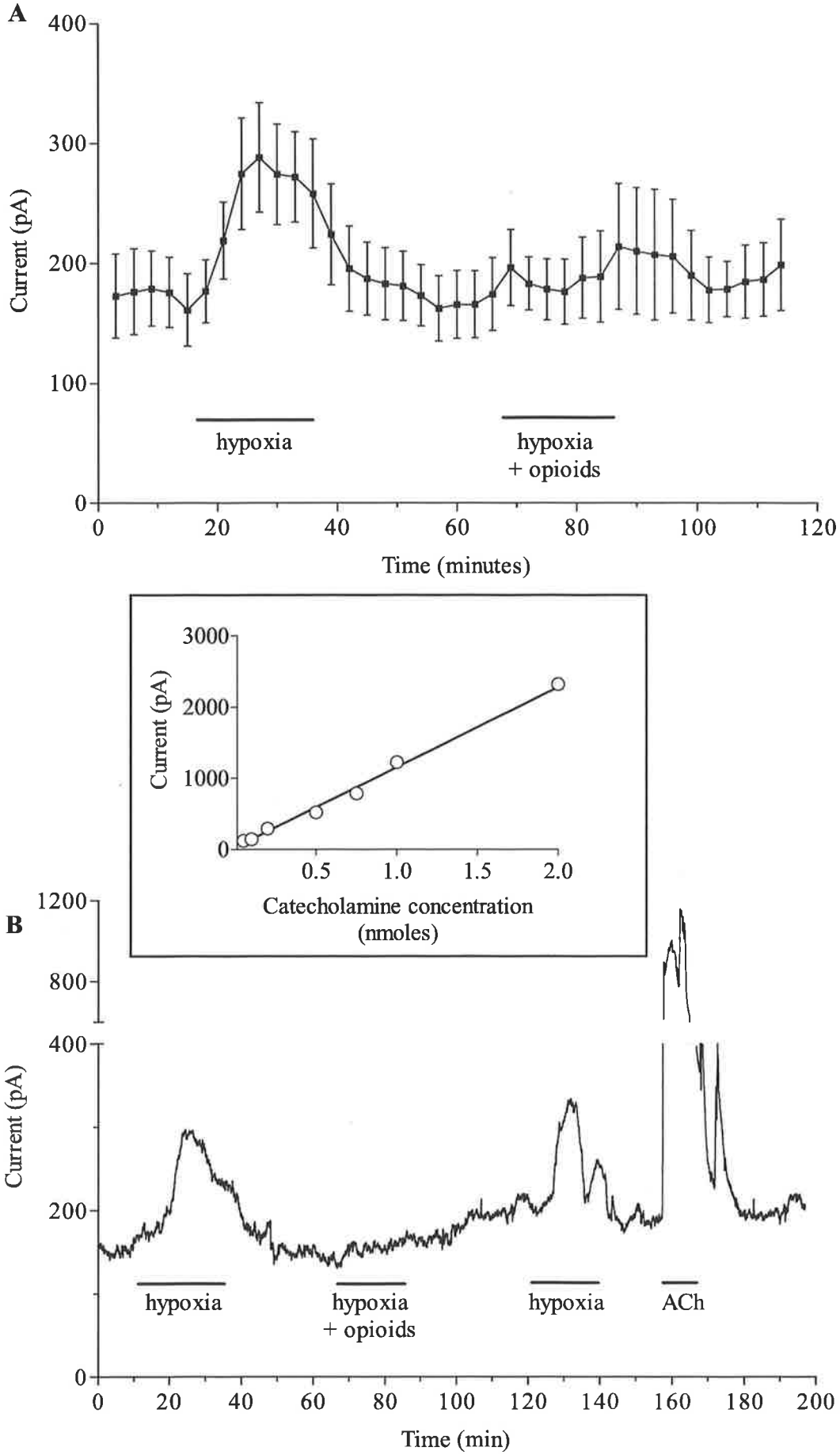
There was a significant difference between the time profile of the catecholamine response to hypoxia alone compared to the catecholamine response to hypoxia in the presence of the opioid agonist i.e.; there was a significant interaction ($p<0.001$) between

the effects of treatment and time. During the control period when normoxic Krebs buffer was being perfused through the gland, the average baseline current amplitude for the 12 minutes prior to exposure to hypoxia was 176.1 ± 15.1 pA (Appendix Fig. 1A; n=5). The adrenal glands were then perfused for 20 minutes with hypoxic Krebs solution which caused a significant rise in catecholamine secretion with a peak reached after approximately 12 minutes exposure to hypoxia causing a rise in current amplitude to 288.3 ± 45.7 pA (n=5, F=10.3, $p<0.001$), an increase in current size of 61%. This increase in secretion was fully reversed upon washout with normoxic solution.

In the presence of the opioid agonists DALDA and U-62066, however, no increase in secretion during hypoxia was seen. The control level from the 12 minutes before exposure to hypoxia and the opioid agonists was 168.0 ± 12.8 pA (n=5), not significantly different from the peak current obtained during hypoxia in the presence of opioid agonists of 196.5 ± 31.7 (n=5).

The effect of μ- and κ-type receptor stimulation on the hypoxic response is reversible once the agonists have been removed as demonstrated by the reapplication of hypoxia causing catecholamine secretion in Appendix Figure 1B. While the increased rate of exocytosis from these perfused adrenals is significant during hypoxia, it is far less than that which is caused by cholinergic stimulation with acetylcholine (100 μM) in this figure.

These results indicate that the hypoxia-evoked secretion of catecholamines from the fetal adrenal gland is suppressed when μ- and κ-type opioid receptors are activated by their respective agonists. The mechanisms underlying this action of opioid receptor activation on the direct hypoxic response is addressed in full in Section 4.


A
400
300
200
100
0
Current (pA)
hypoxia
hypoxia
+ opioids
0
20
40
60
80
100
120
Time (minutes)
3000
2000
1000
0
Current (pA)
0.5
1.0
1.5
2.0
Catecholamine concentration
(nmoles)
B
1200
800
400
200
0
Current (pA)
hypoxia
hypoxia
+ opioids
hypoxia
ACh
0
20
40
60
80
100
120
140
160
180
200
Time (min)

Appendix Figure 1 (previous page): Stimulation of μ- and κ-type opioid receptors diminishes hypoxia-evoked catecholamine secretion from whole perfused fetal adrenal glands. Carbon fibre electrode measurements of current amplitude due to the presence of catecholamines are averaged in **A** in three minute intervals with data represented as means ± SEM (n=5). An example current trace from one experiment is shown in **B**. The word opioids represents the application of DALDA (1 μM) and U-62066 (1 μM) together while ACh indicates application of acetylcholine (100 μM). **Box**: Calibration curve of the carbon fibre electrode measuring the relationship between the current amplitude produced by known molarities of adrenaline.

APPENDIX 2: PUBLICATIONS ARISING FROM THIS THESIS

Keating, D.J., Rychkov, G.Y., and Roberts, M.L. (2001) Oxygen sensitivity in the sheep adrenal medulla: role of SK channels.
American Journal of Physiology, v. 281 (5), pp. C1434-C1441

REFERENCES

Adams, M. B., Simonetta, G. and McMillen, I. C. (1996). The non-neurogenic catecholamine response of the fetal adrenal to hypoxia is dependent on activation of voltage sensitive Ca^{2+} channels. *Developmental Brain Research* **94**: 182-189.

Albertson, B. D., Hobson, W. C., Burnett, B. S., Turner, P. T., Clark, R. V., Schiebinger, R. J., Loriaux, D. L. and Cutler, G. B., Jr. (1984). Dissociation of cortisol and adrenal androgen secretion in the hypophysectomized, adrenocorticotropin-replaced chimpanzee. *Journal of Clinical Endocrinology and Metabolism* **59**: 13-8.

Albillos, A., Artalejo, A. R., Lopez, M. G., Gandia, L., Garcia, A. G. and Carbone, E. (1994). Calcium channel subtypes in cat chromaffin cells. *Journal of Physiology* **477**: 197-213.

Albillos, A., Garcia, A. G., Olivera, B. and Gandia, L. (1996). Re-evaluation of the P/Q Ca^{2+} channel components of Ba^{2+} currents in bovine chromaffin cells superfused with solutions containing low and high Ba^{2+} concentrations. *Pflugers Archives* **432**: 1030-8.

Albillos, A., Neher, E. and Moser, T. (2000). R-Type Ca^{2+} channels are coupled to the rapid component of secretion in mouse adrenal slice chromaffin cells. *Journal of Neuroscience* **20**: 8323-30.

Aldea, M., Jun, K., Shin, H. S., Andres-Mateos, E., Solis-Garrido, L. M., Montiel, C., Garcia, A. G. and Albillos, A. (2002). A perforated patch-clamp study of calcium currents and exocytosis in chromaffin cells of wild-type and α_{1A} knockout mice. *Journal of Neurochemistry* **81**: 911-21.

Archer, S. L., Huang, J., Henry, T., Peterson, D. and Weir, E. K. (1993). A redox-based O_2 sensor in rat pulmonary vasculature. *Circulation Research* **73**: 1100-12.

Artalejo, A. R., Garcia, A. G. and Neher, E. (1993). Small-conductance Ca^{2+}-activated K^+ channels in bovine chromaffin cells. *Pflugers Archives* **423**: 97-103.

Barbara, J.-G. and Takeda, K. (1995). Voltage-dependent currents and modulation of calcium channel expression in zona fasciculata cells from rat adrenal gland. *Journal of Physiology* **488**: 609-622.

Benot, A. R. and Lopez-Barneo, J. (1990). Feedback inhibition of Ca2+ currents by dopamine in glomus cells of the carotid body. *European Journal of Neuroscience* **2**: 809-812.

Birnbaumer, L., Qin, N., Olcese, R., Tareilus, E., Platano, D., Costantin, J. and Stefani, E. (1998). Structures and functions of calcium channel β subunits. *Journal of Bioenergetics and Biomembranes* **30**: 357-75.

Borges, R. (1994). Histamine H1 receptor activation mediates the preferential release of adrenaline in the rat adrenal gland. *Life Science* **54**: 631-40.

Bournaud, R., Hidalgo, J., Yu, H., Jaimovich, E. and Shimahara, T. (2001). Low threshold T-type calcium current in rat embryonic chromaffin cells. *Journal of Physiology* **537**: 35-44.

Brandt, B. L., Hagiwara, S., Kidokoro, Y. and Miyazaki, S. (1976). Action potentials in the rat chromaffin cell and effects of acetylcholine. *Journal of Physiology* **263**: 417-39.

Brown, D. A. and Adams, P. R. (1980). Muscarinic suppression of a novel voltage-sensitive K^+ current in a vertebrate neurone. *Nature* **283**: 673-6.

Buckler, K. J. (1997). A novel oxygen-sensitive potassium current in rat carotid body type 1 cells. *Journal of Physiology* **498**: 649-662.

Buckler, K. J. (1999). Background leak K^+-currents and oxygen sensing in carotid body type 1 cells. *Respiratory Physiology* **115**: 179-87.

Buckler, K. J. and Vaughan-Jones, R. D. (1994). Effects of hypoxia on membrane potential and intracellular calcium in rat neonatal carotid body type 1 cells. *Journal of Physiology* **476**: 423-428.

Buckler, K. J., Williams, B. A. and Honore, E. (2000). An oxygen-, acid- and anaesthetic-sensitive TASK-like background channel in rat arterial chemoreceptor cells. *Journal of Physiology* **525**: 135-142.

Bureau, M. A., Lamarche, J., Foulon, P. and Dalle, D. (1985). The ventilatory response to hypoxia in the newborn lamb after carotid body denervation. *Respiratory Physiology* **60**: 109-19.

Cannon, S. D., Wilson, S. P. and Walsh, K. B. (1994). A G protein-activated K^+ current in bovine adrenal chromaffin cells: possible regulatory role in exocytosis. *Molecular Pharmacology* **45**: 109-16.

Chemin, J., Monteil, A., Perez-Reyes, E., Bourinet, E., Nargeot, J. and Lory, P. (2002). Specific contribution of human T-type calcium channel isotypes ($\alpha_{(1G)}$, $\alpha_{(1H)}$ and $\alpha_{(1I)}$) to neuronal excitability. *Journal of Physiology* **540**: 3-14.

Cheung, C. Y. (1989). Adrenal growth and ontogeny of adrenal substance P, enkephalins and catecholamines in the ovine fetus. *Journal of Developmental Physiology* **12**: 77-84.

Cheung, C. Y. (1990). Fetal adrenal medulla catecholamine response to hypoxia - direct and neural components. *American Journal of Physiology* **258**: R1340-R1346.

Colliver, T. L., Hess, E. J. and Ewing, A. G. (2001). Amperometric analysis of exocytosis at chromaffin cells from genetically distinct mice. *Journal of Neuroscience Methods* **105**: 95-103.

Comline, R. S. and Silver, M. (1961). The release of adrenaline and noradrenaline from the adrenal glands of the fetal sheep. *Journal of Physiology* **156**: 424-444.

Conforti, L. and Millhorn, D. E. (1997). Selective inhibition of a slow-inacivaing voltage-dependent K^+ channel in rat PC12 cells by hypoxia. *Journal of Physiology* **502**: 293-305.

Coupland, R. E. (1989). The natural history of the chromaffin cell - twenty five years on the beginning. *Archives of Histology and Cytology* **52**: 331-341.

Coupland, R. E., Parker, T. L., Kesse, W. K. and Mohamed, A. A. (1989). The innervation of the adrenal gland. III. Vagal innervation. *Journal of Anatomy* **163**: 173-81.

Coupland, R. E., Tomlinson, A., Crowe, J. and Brindley, D. N. (1984). Effects of hypophysectomy and metyrapone on the catecholamine content and volumes of adrenaline- and noradrenaline-storing cells in the rat adrenal medulla. *Journal of Endocrinology* **101**: 345-52.

Cui, Y. and Pun, R. Y. (1994). Angiotensin II suppresses Na^+ currents in bovine adrenal chromaffin cells. *Peptides* **15**: 67-72.

Cutler, G. B., Jr., Davis, S. E., Johnsonbaugh, R. E. and Loriaux, D. L. (1979). Dissociation of cortisol and adrenal androgen secretion in patients with secondary adrenal insufficiency. *Journal of Clinical Endocrinology and Metabolism* **49**: 604-9.

Cutz, E., Gillian, J. and Track, N. (1984). Pulmonary endocrine cells in developing human lung and during neonatal adaptation. The Endocrine Lung in Health and Disease. Becker and Gazdar. Philadelphia, Saunders: 210-231.

Cutz, E. and Jackson, A. (1999). Neuroepithelial bodies as airway oxygen sensors. *Respiratory Physiology* **115**: 201-14.

Dawes, G. S., Lewis, B. V., Milligan, J. E., Roach, M. R. and Talner, N. S. (1968). Vasomotor responses in the hind limbs of foetal and new-born lambs to asphyxia and aortic chemoreceptor stimulation. *Journal of Physiology* **195**: 55-81.

Diverse-Pierluissi, M., Dunlap, K. and Westhead, E. W. (1991). Multiple actions of extracellular ATP on calcium currents in cultured bovine chromaffin cells. *Proceedings of the National Academy of Science U S A* **88**: 1261-5.

Donnelly, D. F. (1995). Does catecholamine secretion mediate the hypoxia-induced increase in nerve activity? *Biological Signals* **4**: 304-9.

Doroshenko, P. A., Woppmann, A., Miljanich, G. and Augustine, G. J. (1997). Pharmacologically distinct presynaptic calcium channels in cerebellar excitatory and inhibitory synapses. *Neuropharmacology* **36**: 865-72.

Duchen, M. and Biscoe, T. (1992). Relative mitochondrial membrane potential and $[Ca^{2+}]_i$ in type I cells isolated from the rabbit carotid body. *Journal of Physiology* **450**: 33-61.

Dutar, P., Rascol, O. and Lamour, Y. (1989). ω-conotoxin GVIA blocks synaptic transmission in the CA1 field of the hippocampus. *European Journal of Physiology* **174**: 261-6.

Ellinor, P. T., Zhang, J. F., Randall, A. D., Zhou, M., Schwarz, T. L., Tsien, R. W. and Horne, W. A. (1993). Functional expression of a rapidly inactivating neuronal calcium channel. *Nature* **363**: 455-8.

Fenwick, E. M., Fajdiga, P. B., Howe, N. B. and Livett, B. G. (1978). Functional and morphological characterization of isolated bovine adrenal medullary cells. *Journal of Cell Biology* **76**: 12-30.

Fidone, S. J., Gonzalez, C., Dinger, B. G. and Hanson, G. R. (1988). Mechanisms of chemotransmission in the mammalian carotid body. *Progressive Brain Research* **74**: 169-79.

Fisher, T. E. and Bourque, C. W. (2001). The function of Ca^{2+} channel subtypes in exocytotic secretion: new perspectives from synaptic and non-synaptic release. *Progress in Biophysics and Molecular Biology* **77**: 269-303.

Fitzgerald, R. S. and Shirahata, M. (1994). Acetylcholine and carotid body excitation during hypoxia in the cat. *Journal of Applied Physiology* **76**: 1566-74.

Fitzgerald, R. S., Shirahata, M. and Wang, H. Y. (1999). Acetylcholine release from cat carotid bodies. *Brain Research* **841**: 53-61.

Fitzgerald, R. S., Shirahata, M. and Wang, H. Y. (2000). Acetylcholine is released from *in vitro* cat carotid bodies during hypoxic stimulation. *Advances in Experimental and Medical Biology* **475**: 485-94.

Foucart, S., de Champlain, J. and Nadeau, R. (1991). Modulation by β-adrenoceptors and angiotensin II receptors of splanchnic nerve evoked catecholamine release from the adrenal medulla. *Canadian Journal of Physiology and Pharmacology* **69**: 1-7.

Fu, X. W., Nurse, C. A., Wang, Y. T. and Cutz, E. (1999). Selective modulation of membrane currents by hypoxia in intact airway chemoreceptors from neonatal rabbit. *Journal of Physiology* **514**: 139-50.

Fu, X. W., Nurse, C. A., Wong, V. and Cutz, E. (2002). Hypoxia-induced secretion of serotonin from intact pulmonary neuroepithelial bodies in neonatal rabbit. *Journal of Physiology* **539**: 503-10.

Gandia, L., Borges, R., Albillos, A. and Garcia, A. G. (1995). Multiple calcium channel subtypes in isolated rat chromaffin cells. *Pflugers Archives* **430**: 55-63.

Gandia, L., Garcia, A. G. and Morad, M. (1993). ATP modulation of calcium channels in chromaffin cells. *Journal of Physiology* **470**: 55-72.

Gandia, L., Mayorgas, I., Michelena, P., Cuchillo, I., de Pascual, R., Abad, F., Novalbos, J. M., Larranaga, E. and Garcia, A. G. (1998). Human adrenal chromaffin cell calcium channels: drastic current facilitation in cell clusters, but not in isolated cells. *Pflugers Archives* **436**: 696-704.

Ganfornina, M. D. and Lopez-Barneo, J. (1991). Single K^+ channels in membrane patches of arterial chemoreceptor cells are modulated by O_2 tension. *Proceedings of the National Academy of Science of the USA* **88**: 2927-30.

Ganfornina, M. D. and Lopez-Barneo, J. (1992). Potassium channel types in arterial chemoreceptor cells and their selective modulation by oxygen. *Journal of General Physiology* **100**: 401-426.

Gao, B., Sekido, Y., Maximov, A., Saad, M., Forgacs, E., Latif, F., Wei, M. H., Lerman, M., Lee, J. H., Perez-Reyes, E., Bezprozvanny, I. and Minna, J. D. (2000). Functional properties of a new voltage-dependent calcium channel $\alpha_2\delta$ auxiliary subunit gene (CACNA2D2). *Journal of Biological Chemistry* **275**: 12237-42.

Garcia-Palomero, E., Cuchillo-Ibanez, I., Garcia, A. G., Renart, J., Albillos, A. and Montiel, C. (2000). Greater diversity than previously thought of chromaffin cell Ca^{2+} channels, derived from mRNA identification studies. *FEBS Letters* **481**: 235-9.

Gelband, C. H. and Gelband, H. (1997). Ca^{2+} release from intracellular stores is an initial step in hypoxic pulmonary vasoconstriction of rat pulmonary artery resistance vessels. *Circulation* **96**: 3647-54.

Gonzalez, C., Almaraz, L., Obeso, A. and Rigual, R. (1994). Carotid body chemoreceptors: from natural stimuli to sensory discharges. *Physiology Reviews* **74**: 829-98.

Gonzalez, C., Almarez, L. and Fidone, S. (1977). Increased release of ^{3}H-dopamine during low O_2 stimulation of rabbit carotid body *in vitro*. *Neuroscience Letters* **6**: 95-99.

Gurney, A. M., Osipenko, O. N., MacMillan, D. and Kempsill, F. E. (2002). Potassium channels underlying the resting potential of pulmonary artery smooth muscle cells. *Clinical and experimental pharmacology and physiology* **29**: 330-3.

Harder, D. R., Madden, J. A. and Dawson, C. (1985). Hypoxic induction of Ca^{2+}-dependent action potentials in small pulmonary arteries of the cat. *Journal of Applied Physiology* **59**: 1389-93.

Hartness, M. E., Lewis, A., Searle, G. J., O'Kelly, I., Peers, C. and Kemp, P. J. (2001). Combined antisense and pharmacological approaches implicate hTASK as an airway O_2 sensing K^+ channel. *Journal of Biological Chemistry* **276**: 26499-508.

Hatton, C. J., Carpenter, E., Pepper, D. R., Kumar, P. and Peers, C. (1997). Developmental changes in isolated rat type I carotid body cell K^+ currents and their modulation by hypoxia. *Journal of Physiology* **501**: 49-58.

Hernandez-Guijo, J. M., de Pascual, R., Garcia, A. G. and Gandia, L. (1998). Separation of calcium channel current components in mouse chromaffin cells superfused with low- and high-barium solutions. *Pflugers Archives* **436**: 75-82.

Ho, W. H., Kao, M. H., Huang, B. N., Chang, H. C. and Lu, K. S. (1994). Small granule-containing (SGC) cells in the hamster adrenal medulla. *Gaoxiong Yi Xue Ke Xue Za Zhi* **10**: 28-34.

Holets, V. and Elde, R. (1982). The differential distribution and relationship of serotoninergic and peptidergic fibers to sympathoadrenal neurons in the intermediolateral cell column of the rat: a combined retrograde axonal transport and immunofluorescence study. *Neuroscience* **7**: 1155-74.

Holgert, H., Aman, K., Cozzari, C., Hartman, B. K., Brimijoin, S., Emson, P., Goldstein, M. and Hokfelt, T. (1995). The cholinergic innervation of the adrenal gland and its relation to enkephalin and nitric oxide synthase. *Neuroreport* **6**: 2576-80.

Holgert, H., Dagerlind, A. and Hokfelt, T. (1996). Phenotype of intraadrenal ganglion neurons during postnatal development in rat. *Journal of Comparitive Neurology* **371**: 603-20.

Holgert, H., Dagerlind, A. and Hokfelt, T. (1998). Immunohistochemical characterization of the peptidergic innervation of the rat adrenal gland. *Hormone and Metabolism Research* **30**: 315-22.

Hollins, B. and Ikeda, S. R. (1996). Inward currents underlying action potentials in rat adrenal chromaffin cells. *Journal of Neurophysiology* **76**: 1195-211.

Hollinshead, W. H. (1937). The innervation of the abdominal chromaffin tissue. *Journal of Comparitive Neurology* **67**: 133-143.

Horne, A. L. and Kemp, J. A. (1991). The effect of ω-conotoxin GVIA on synaptic transmission within the nucleus accumbens and hippocampus of the rat in vitro. *British Journal of Pharmacology* **103**: 1733-9.

Huguenard, J. R. (1996). Low-threshold calcium currents in central nervous system neurons. *Annual Review of Physiology* **58**: 329-48.

Hulme, J. T., Coppock, E. A., Felipe, A., Martens, J. R. and Tamkun, M. M. (1999). Oxygen sensitivity of cloned voltage-gated K^+ channels expressed in the pulmonary vasculature. *Circulation Research* **85**: 489-97.

Inoue, M., Fujishiro, N. and Imanaga, I. (1998). Hypoxia and cyanide induce depolarization and catecholamine release in dispersed guinea-pig chromaffin cells. *Journal of Physiology* **507**: 807-18.

Inoue, M. and Kuriyama, H. (1990). Muscarine induces two distinct current responses in adrenal chromaffin cells of the guinea-pig. *Japanese Journal of Physiology* **40**: 679-91.

Inoue, M., Sakamoto, Y. and Imanaga, I. (1995). Phosphatidylinositol hydrolysis is involved in production of Ca^{2+}-dependent currents, but not non-selective cation currents, by muscarine in chromaffin cells. *European Journal of Pharmacology* **276**: 123-9.

Itskovitz, J., Goetzman, B. W. and Rudolph, A. M. (1982). Effects of hemorrhage on umbilical venous return and oxygen delivery in fetal lambs. *American Journal of Physiology* **242**: H543-8.

Itskovitz, J., LaGamma, E. F. and Rudolph, A. M. (1987). Effects of cord compression on fetal blood flow distribution and O_2 delivery. *American Journal of Physiology* **252**: H100-9.

Jones, C. T. and Ritchie, J. W. (1983). The effects of adrenergic blockade on fetal response to hypoxia. *Journal of Developmental Physiology* **5**: 211-22.

Jones, C. T. and Robinson, R. O. (1975). Plasma catecholamines in foetal and adult sheep. *Journal of Physiology* **248**: 15-33.

Kajihara, H., Akimoto, T. and Iijima, S. (1978). On the chromaffin cells in dog adrenal medulla; with special reference to the small granule chromaffin cells (SGC cells). *Cell Tissue Research* **191**: 1-14.

Kamiya, H., Sawada, S. and Yamamoto, C. (1988). Synthetic ω-conotoxin blocks synaptic transmission in the hippocampus in vitro. *Neuroscience Letters* **91**: 84-8.

Kesse, W. K., Parker, T. L. and Coupland, R. E. (1988). The innervation of the adrenal gland. I. The source of pre- and postganglionic nerve fibres to the rat adrenal gland. *Journal of Anatomy* **157**: 33-41.

Kim, D. K., Oh, E. K., Summers, B. A., Prabhakar, N. R. and Kumar, G. K. (2001). Release of substance P by low oxygen in the rabbit carotid body: evidence for the involvement of calcium channels. *Brain Research* **892**: 359-69.

Kim, S. J., Lim, W. and Kim, J. (1995). Contribution of L- and N-type calcium currents to exocytosis in rat adrenal medullary chromaffin cells. *Brain Research* **675**: 289-96.

Kitamura, K., Houchi, H., Yoshizumi, M., Matsumoto, K. and Oka, M. (1996). Potentiation by apamin of histamine-stimulated catecholamine biosynthesis and tyrosine hydroxylase phosphorylation in cultured bovine adrenal chromaffin cells. *Journal of Experimental Medicine* **43**: 17-23.

Kitamura, N., Ohta, T., Ito, S. and Nakazato, Y. (1997). Calcium channel subtypes in porcine adrenal chromaffin cells. *Pflugers Archives* **434**: 179-87.

Kobayashi, S. and Coupland, R. E. (1993). Morphological aspects of chromaffin tissue: the differential fixation of adrenaline and noradrenaline. *Journal of Anatomy* **183**: 223-35.

Lahiri, S., Roy, A., Rozanov, C. and Mokashi, A. (1998). K^+-current modulated by Po_2 in type I cells in rat carotid body is not a chemosensor. *Brain Research* **794**: 162-5.

Lai, G. J. and McCobb, D. P. (2002). Opposing actions of adrenal androgens and glucocorticoids on alternative splicing of *Slo* potassium channels in bovine chromaffin cells. *Proceedings of the National Academy of Science of the USA* **99**: 7722-7.

Lang, R. J., Harvey, J. R. and Watson, M. J. (2002). Thiol reagents and nitric oxide modulate the gating of BK_{Ca} channels from the guinea-pig taenia caeci

Effects of nitric oxide donors, S-nitroso-L-cysteine and sodium nitroprusside, on the whole-cell and single channel currents in single myocytes of the guinea-pig proximal colon. *Clinical and experimental pharmacology and physiology* **29**: 944-9.

Lang, R. J. and Watson, M. J. (1998). Effects of nitric oxide donors, S-nitroso-L-cysteine and sodium nitroprusside, on the whole-cell and single channel currents in single myocytes of the guinea-pig proximal colon. *British Journal of Pharmacology* **123**: 505-17.

Langley, K. and Grant, N. J. (1999). Molecular markers of sympathoadrenal cells. *Cell Tissue Research* **298**: 185-206.

Lara, B., Gandia, L., Martinez-Sierra, R., Torres, A. and Garcia, A. G. (1998). Q-type Ca^{2+} channels are located closer to secretory sites than L-type channels: functional evidence in chromaffin cells. *Pflugers Archives* **435**: 472-8.

Lara, B., Zapater, P., Montiel, C., de la Fuente, M. T., Martinez-Sierra, R., Ballesta, J. J., Gandia, L. and Garcia, A. G. (1995). Density of apamin-sensitive Ca^{2+}-dependent K^{+} channels in bovine chromaffin cells: relevance to secretion. *Biochemical Pharmacology* **49**: 1459-68.

Lauweryns, J. M. and Cokelaere, M. (1973). Hypoxia-sensitive neuro-epithelial bodies. Intrapulmonary secretory neuroreceptors, modulated by the CNS. *Zeitschrift f r Zellforschung und mikroskopische Anatomie* **145**: 521-40.

Leach, R. M., Hill, H. M., Snetkov, V. A., Robertson, T. P. and Ward, J. P. (2001). Divergent roles of glycolysis and the mitochondrial electron transport chain in hypoxic pulmonary vasoconstriction of the rat: identity of the hypoxic sensor. *Journal of Physiology* **536**: 211-24.

Lee, J., Lim, W., Eun, S. Y., Kim, S. J. and Kim, J. (2000). Inhibition of apamin-sensitive K^+ current by hypoxia in adult rat adrenal chromaffin cells. *Pflugers Archives* **439**: 700-4.

Lewis, A., Hartness, M. E., Chapman, C. G., Fearon, I. M., Meadows, H. J., Peers, C. and Kemp, P. J. (2001). Recombinant hTASK1 is an O_2-sensitive K^+ channel. *Biochemical and Biophysical Research Communications* **285**: 1290-4.

Lewis, A., Peers, C., Ashford, M. L. and Kemp, P. J. (2002). Hypoxia inhibits human recombinant large conductance, Ca^{2+}-activated K^+ (maxi-K) channels by a mechanism which is membrane delimited and Ca^{2+} sensitive. *Journal of Physiology* **540**: 771-780.

Livett, B. G. and Marley, P. D. (1986). Effects of opioid peptides and morphine on histamine-induced catecholamine secretion from cultured, bovine adrenal chromaffin cells. *British Journal of Pharmacology* **89**: 327-34.

Llinas, R., Sugimori, M., Lin, J. W. and Cherksey, B. (1989). Blocking and isolation of a calcium channel from neurons in mammals and cephalopods utilizing a toxin fraction (FTX) from funnel-web spider poison. *Proceedings of the National Academy of Science of the USA* **86**: 1689-93.

Lomax, R. B., Michelena, P., Nunez, L., Garcia-Sancho, J., Garcia, A. G. and Montiel, C. (1997). Different contributions of L- and Q-type Ca^{2+} channels to Ca^{2+} signals and secretion in chromaffin cell subtypes. *American Journal of Physiology* **272**: C476-84.

Lopez, M. G., Artalejo, A. R., Garcia, A. G., Neher, E. and Garcia-Sancho, J. (1995). Veratridine-induced oscillations of cytosolic calcium and membrane potential in bovine chromaffin cells. *Journal of Physiology* **482**: 15-27.

Lopez-Barneo, J., Benot, A.R. and Urena, J. (1993). Oxygen sensing and the electrophysiology of arterial chemoreceptor cells. *News in Physiological Science* **8**: 191-195.

Lopez-Barneo, J., Lopez-Lopez, J. R., Urena, J. and Gonzalez, C. (1988). Chemotransduction in the carotid body: K^+ current modulated by Po_2 in type 1 chemoreceptor cells. *Science* **241**: 580-582.

Lopez-Lopez, J., Gonzalez, C., Urena, J. and Lopez-Barneo, J. (1989). Low pO_2 selectively inhibits K channel activity in chemoreceptor cells of the mammalian carotid body. *Journal of General Physiology* **93**: 1001-15.

Lovell, P. V., James, D. G. and McCobb, D. P. (2000). Bovine versus rat adrenal chromaffin cells: big differences in BK potassium channel properties. *Journal of Neurophysiology* **83**: 3277-86.

Lovell, P. V. and McCobb, D. P. (2001). Pituitary control of BK potassium channel function and intrinsic firing properties of adrenal chromaffin cells. *Journal of Neuroscience* **21**: 3429-42.

Madden, J. A., Dawson, C. A. and Harder, D. R. (1985). Hypoxia-induced activation in small isolated pulmonary arteries from the cat. *Journal of Applied Physiology* **59**: 113-8.

Marley, P. D., Cheung, C. Y., Thomson, K. A. and Murphy, R. (1996). Activation of tyrosine hydroxylase by pituitary adenylate cyclase-activating polypeptide (PACAP-27) in bovine adrenal chromaffin cells. *Journal of the Autonomic Nervous System* **60**: 141-6.

Martin, R. L., Lee, J. H., Cribbs, L. L., Perez-Reyes, E. and Hanck, D. A. (2000). Mibefradil block of cloned T-type calcium channels. *J Pharmacol Exp Ther* **295**: 302-8.

Marty, A. and Neher, E. (1985). Potassium channels in cultured bovine adrenal chromaffin cells. *Journal of Physiology* **367**: 117-141.

McCobb, D. P. and Beam, K. G. (1991). Action potential waveform voltage-clamp commands reveal striking differences in calcium entry via low and high voltage-activated calcium channels. *Neuron* **7**: 119-27.

McKenzie, S. and Marley, P. D. (2002). Caffeine stimulates Ca^{2+} entry through store-operated channels to activate tyrosine hydroxylase in bovine chromaffin cells. *European Journal of Neuroscience* **15**: 1485-92.

McMillen, I. M., Mulvogue, H. M., Coulter, C. L., Browne, C. A. and Howe, P. R. C. (1988). Ontogeny of catecholamine-synthesising enzymes and enkephalins in sheep adrenal medulla: an immunocytochemical study. *Journal of Endocrinology* **118**: 221-226.

McNicol, A. M. (1992). The human adrenal gland. Aspects of structure, function and pathology. In *The Adrenal Gland.* New York, Raven Press.

Mintz, I. M., Venema, V. J., Swiderek, K. M., Lee, T. D., Bean, B. P. and Adams, M. E. (1992). P-type calcium channels blocked by the spider toxin ω-Aga-IVA. *Nature* **355**: 827-9.

Mochizuki-Oda, N., Takeuchi, Y., Matsamura, K., Oosawa, Y., and Watanabe, Y. (1997). Hypoxia-induced catecholamine release and intracellular Ca^{2+} increase via suppression of K^+ channels in cultured rat adrenal chromaffin cells. *Journal of Neurochemistry* **69**: 377-387.

Mojet, M. H., Mills, E. and Duchen, M. R. (1997). Hypoxia-induced catecholamine secretion in isolated newborn rat adrenal chromaffin cells is mimicked by inhibition of mitochondrial respiration. *Journal of Physiology* **504**: 175-89.

Montiel, C., Lopez, M. G., Sanchez-Garcia, P., Maroto, R., Zapater, P. and Garcia, A. G. (1995). Contribution of SK and BK channels in the control of catecholamine release by electrical stimulation of the cat adrenal gland. *Journal of Physiology* **486**: 427-37.

Nagayama, T., Koshika, T., Hisa, H., Kimura, T. and Satoh, S. (1997). Apamin-sensitive SK_{Ca} channels modulate adrenal catecholamine release in anesthetized dogs. *European Journal of Pharmacology* **327**: 135-41.

Nagayama, T., Yoshida, M., Suzuki-Kusaba, M., Hisa, H., Kimura, T. and Satoh, S. (1998). The role of BK_{Ca} channels in the nitric oxide-mediated regulation of adrenal catecholamine secretion. *European Journal of Pharmacology* **353**: 169-76.

Nakayama, K. (1961). Surgical removal of the carotid body for bronchial asthma. *Chest* **40**: 595-604.

Neely, A. and Lingle, C. J. (1992). Two components of calcium-activated potassium current in rat adrenal chromaffin cells. *Journal of Physiology* **453**: 97-131.

Newcomb, R., Szoke, B., Palma, A., Wang, G., Chen, X., Hopkins, W., Cong, R., Miller, J., Urge, L., Tarczy-Hornoch, K., Loo, J. A., Dooley, D. J., Nadasdi, L., Tsien, R. W., Lemos, J. and Miljanich, G. (1998). Selective peptide antagonist of the class E calcium channel from the venom of the tarantula *Hysterocrates gigas*. *Biochemistry* **37**: 15353-62.

Nowycky, M. C., Fox, A. P. and Tsien, R. W. (1985). Long-opening mode of gating of neuronal calcium channels and its promotion by the dihydropyridine calcium agonist Bay K 8644. *Proceedings of the National Academy of Science USA* **82**: 2178-82.

O'Farrell, M., Ziogas, J. and Marley, P. D. (1997). Effects of N- and L-type calcium channel antagonists and (+/-)-Bay K8644 on nerve-induced catecholamine secretion from bovine perfused adrenal glands. *British Journal of Pharmacology* **121**: 381-8.

O'Kelly, I., Peers, C. and Kemp, P. J. (1998). O_2-sensitive K^+ channels in neuroepithelial body-derived small cell carcinoma cells of the human lung. *American Journal of Physiology* **275**: L709-16.

O'Kelly, I., Stephens, R. H., Peers, C. and Kemp, P. J. (1999). Potential identification of the O_2-sensitive K^+ current in a human neuroepithelial body-derived cell line. *American Journal of Physiology* **276**: L96-L104.

Osanai, S., Buerk, D. G., Mokashi, A., Chugh, D. K. and Lahiri, S. (1997). Cat carotid body chemosensory discharge *in vitro* is insensitive to charybdotoxin. *Brain Research* **747**: 324-7.

Osipenko, O. N., Tate, R. J. and Gurney, A. M. (2000). Potential role for Kv3.1b channels as oxygen sensors. *Circulation Research* **86**: 534-40.

Pardal, R., Ludewig, U., Garcia-Hirschfeld, J. and Lopez-Barneo, J. (2000). Secretory responses of intact glomus cells in thin slices of rat carotid body to hypoxia and tetraethylammonium. *Proceedings of the National Academy of Science of the USA* **97**: 2361-2366.

Parker, T. L., Kesse, W. K., Mohamed, A. A. and Afework, M. (1993). The innervation of the mammalian adrenal gland. *Journal of Anatomy* **183**: 265-76.

Parker, T. L., Mohamed, A. A. and Coupland, R. E. (1990). The innervation of the adrenal gland. IV. The source of pre- and postganglionic nerve fibres to the guinea-pig adrenal gland. *Journal of Anatomy* **172**: 17-24.

Peers, C. (1990). Hypoxic suppression of K^+ currents in type I carotid body cells: selective effect on the Ca^{2+}-activated K^+ current. *Neuroscience Letters* **119**: 253-256.

Peers, C. and Kemp, P. J. (2001). Acute oxygen sensing: diverse but convergent mechanisms in airway and arterial chemoreceptors. *Respiratory Research* **2**: 145-9.

Perez-Garcia, M. T., Lopez-Lppez, J. R., Riesco, A. M., Hoppe, U. C., Marban, E., Gonzalez, C. and Johns, D. C. (2000). Viral gene transfer of dominant-negative Kv4 construct suppresses an O_2-sensitive K+ current in chemoreceptor cells. *Journal of Neuroscience* **20**: 5689-95.

Peterson, B. Z., DeMaria, C. D., Adelman, J. P. and Yue, D. T. (1999). Calmodulin is the Ca^{2+} sensor for Ca^{2+} -dependent inactivation of L-type calcium channels. *Neuron* **22**: 549-58.

Plattner, H., Artalejo, A. R. and Neher, E. (1997). Ultrastructural organization of bovine chromaffin cell cortex-analysis by cryofixation and morphometry of aspects pertinent to exocytosis. *Journal of Cell Biology* **139**: 1709-17.

Powell, A. D., Teschemacher, A. G. and Seward, E. P. (2000). P2Y purinoceptors inhibit exocytosis in adrenal chromaffin cells via modulation of voltage-operated calcium channels. *Journal of Neuroscience* **20**: 606-16.

Prabhakar, N. R. (1994). Neurotransmitters in the carotid body. *Advances in Experimental and Medical Biology* **360**: 57-69.

Prabhakar, N. R. (2000). Oxygen sensing by the carotid body chemoreceptors. *Journal of Applied Physiology* **88**: 2287-2295.

Prakriya, M., Solaro, C. R. and Lingle, C. J. (1996). $[Ca^{2+}]_i$ elevations detected by BK channels during Ca^{2+} influx and muscarine-mediated release of Ca^{2+} from intracellular stores in rat chromaffin cells. *Journal of Neuroscience* **16**: 4344-59.

Rashid, A. J., Morales, E., Turner, R. W. and Dunn, R. J. (2001). The contribution of dendritic Kv3 K^+ channels to burst threshold in a sensory neuron. *Journal of Neuroscience* **21**: 125-35.

Reuss, M. L., Parer, J. T., Harris, J. L. and Krueger, T. R. (1982). Hemodynamic effects of α-adrenergic blockade during hypoxia in fetal sheep. *American Journal of Obstetrics and Gynecology* **142**: 410-5.

Reuss, M. L. and Rudolph, A. M. (1980). Distribution and recirculation of umbilical and systemic venous blood flow in fetal lambs during hypoxia. *Journal of Developmental Physiology* **2**: 71-84.

Riesco-Fagundo, A. M., Perez-Garcia, M. T., Gonzalez, C. and Lopez-Lopez, J. R. (2001). O_2 modulates large-conductance Ca^{2+}-dependent K^+ channels of rat chemoreceptor cells by a membrane-restricted and CO-sensitive mechanism. *Circulation Research* **89**: 430-6.

Robinson, P. M., Perry, R. A., Hardy, K. J., Coghlan, J. P. and Scoggins, B. A. (1977). The innervation of the adrenal cortex in the sheep, *Ovis ovis*. *Journal of Anatomy* **124**: 117-29.

Robitaille, R., Adler, E. M. and Charlton, M. P. (1990). Strategic location of calcium channels at transmitter release sites of frog neuromuscular synapses. *Neuron* **5**: 773-9.

Rychkov, G. Y., Adams, M. B., McMillen, I. C. and Roberts, M. L. (1998). Oxygen sensing mechanisms are present in the chromaffin cells of the sheep adrenal medulla before birth. *Journal of Physiology* **509**: 887-893.

Salapatek, A. M., Ji, J. and Diamant, N. E. (2002). Ion channel diversity in the feline smooth muscle esophagus. *American Journal of Physiology Gastrointestinal and Liver Physiology* **282**: G288-99.

Salvaterra, C. G. and Goldman, W. F. (1993). Acute hypoxia increases cytosolic calcium in cultured pulmonary arterial myocytes. *American Journal of Physiology* **264**: L323-8.

Sanchez, D., Lopez-Lopez, J. R., Perez-Garcia, M. T., Sanz-Alfayate, G., Obeso, A., Ganfornina, M. D. and Gonzalez, C. (2002). Molecular identification of Kvα subunits that contribute to the oxygen-sensitive K^+ current of chemoreceptor cells of the rabbit carotid body. *Journal of Physiology* **542**: 369-382.

Santana, F., Michelena, P., Jaen, R., Garcia, A. G. and Borges, R. (1999). Calcium channel subtypes and exocytosis in chromaffin cells: a different view from the intact rat adrenal. *Naunyn Schmiedebergs Archives of Pharmacology* **360**: 33-7.

Seidler, F. J. and Slotkin, T. A. (1985). Adrenomedullary function in the neonatal rat: responses to acute hypoxia. *Journal of Physiology* **358**: 1-16.

Seidler, F. J. and Slotkin, T. A. (1986a). Non-neurogenic adrenal catecholamine release in the neonatal rat: exocytosis or diffusion? *Brain Research* **393**: 274-7.

Seidler, F. J. and Slotkin, T. A. (1986b). Ontogeny of adrenomedullary responses to hypoxia and hypoglycemia: role of splanchnic innervation. *Brain Research Bulletin* **16**: 11-14.

Slotkin, T. A. and Seidler, F. J. (1988). Adrenomedullary catecholamine release in the fetus and newborn: secretory mechanisms and their role in stress and survival. *Journal of Developmental Physiology* **10**: 1-16.

Solaro, C. R., Prakriya, M., Ding, J. P. and Lingle, C. J. (1995). Inactivating and noninactivating Ca^{2+}- and voltage-dependent K^{+} current in rat adrenal chromaffin cells. *Journal of Neuroscience* **15**: 6110-23.

Stea, A., Jackson, A. and Nurse, C. A. (1992). Hypoxia and N^6,$O^{2'}$-dibutyryladenosine 3',5'-cyclic monophosphate, but not nerve growth factor, induce Na^{+} channels and hypertrophy in chromaffin-like arterial chemoreceptors. *Proceedings of the National Academy of Science U S A* **89**: 9469-73.

Suh, B. C. and Hille, B. (2002). Recovery from muscarinic modulation of M current channels requires phosphatidylinositol 4,5-bisphosphate synthesis. *Neuron* **35**: 507-20.

Sweeney, M. and Yuan, J. X. (2000). Hypoxic pulmonary vasoconstriction: role of voltage-gated potassium channels. *Respiratory Research* **1**: 40-8.

Takeuchi, Y., Mochizuki-Oda, N., Yamada, H., Kurokawa, K. and Watanabe, Y. (2001). Nonneurogenic hypoxia sensitivity in rat adrenal slices. *Biochemical and Biophysical Research Communications* **289**: 51-6.

Teitel, D. and Rudolph, A. M. (1985). Perinatal oxygen delivery and cardiac function. *Advances in Pediatrics* **32**: 321-347.

Thompson, R. J., Jackson, A. and Nurse, C. A. (1997). Developmental loss of hypoxic chemosensitivity in rat adrenomedullary chromaffin cells. *Journal of Physiology* **498**: 503-510.

Thompson, R. J. and Nurse, C. A. (1998). Anoxia differentially modulates multiple K^+ currents and depolarizes neonatal rat adrenal chromaffin cells. *Journal of Physiology* **512**: 421-34.

Thornburg, K. L. (1991). Fetal response to intrauterine stress. *Ciba Foundation Symposium* **156**: 17-37.

Uceda, G., Artalejo, A. R., de la Fuente, M. T., Lopez, M. G., Albillos, A., Michelena, P., Garcia, A. G. and Montiel, C. (1994). Modulation by L-type Ca^{2+} channels and apamin-sensitive K^+ channels of muscarinic responses in cat chromaffin cells. *American Journal of Physiology* **266**: C1432-9.

Uceda, G., Artalejo, A. R., Lopez, M. G., Abad, F., Neher, E. and Garcia, A. G. (1992). Ca^{2+}-activated K^+ channels modulate muscarinic secretion in cat chromaffin cells. *Journal of Physiology* **454**: 213-30.

Unsicker, K. (1971). On the innervation of the rat and pig adrenal cortex. *Z Zellforsch Mikrosk Anat* **116**: 151-6.

Urena, J., Fernandez-Chacon, R., Benot, A. R., Alvarez de Toledo, G. A. and Lopez-Barneo, J. (1994). Hypoxia induces voltage-dependent Ca^{2+} entry and quantal dopamine secretion in carotid body glomus cells. *Proceedings of the National Academy of Science of the USA* **91**: 10208-11.

Vega-Saenz de Miera, E. and Rudy, B. (1992). Modulation of K^+ channels by hydrogen peroxide. *Biochemical and Biophysical Research Communications* **186**: 1681-1687.

Vergara, C., Latorre, R., Marrion, N. V. and Adelman, J. P. (1998). Calcium-activated potassium channels. *Current Opinions in Neurobiology* **8**: 321-9.

Wada, A., Urabe, M., Yuhi, T., Yamamoto, R., Yanagita, T., Niina, H. and Kobayashi, H. (1995). Large- and small-conductance Ca^{2+}-activated K^+ channels: their role in the nicotinic receptor-mediated catecholamine secretion in bovine adrenal medulla. *Naunyn Schmiedebergs Archives of Pharmacology* **352**: 545-9.

Wang, D., Youngson, C., Wong, V., Yeger, H., Dinauer, M. C., Vega-Saenz Miera, E., Rudy, B. and Cutz, E. (1996). NADPH-oxidase and a hydrogen peroxide-sensitive K^+ channel may function as an oxygen sensor complex in airway chemoreceptors and small cell lung carcinoma cell lines. *Proceedings of the National Academy of Science USA* **93**: 13182-7.

Wang, H. S., Pan, Z., Shi, W., Brown, B. S., Wymore, R. S., Cohen, I. S., Dixon, J. E. and McKinnon, D. (1998). KCNQ2 and KCNQ3 potassium channel subunits: molecular correlates of the M-channel. *Science* **282**: 1890-3.

Watanabe, T., Hiramatsu, K., Ohmori, Y. and Paik, Y. K. (1990). Histo- and cytochemical studies on the distribution of acetylcholinesterase-positive nerve fibers in the goat adrenal gland. *Anatomy, Histology and Embryology* **19**: 245-54.

Wyatt, C. N. and Peers, C. (1995). Ca^{2+}-activated K^+ channels in isolated type I cells of the neonatal rat carotid body. *Journal of Physiology* **483**: 559-65.

Wyatt, C. N., Wright, C., Bee, D. and Peers, C. (1995). O_2-sensitive K^+ currents in carotid body chemoreceptor cells from normoxic and chronically hypoxic rats and their roles in hypoxic chemotransduction. *Proceedings of the National Academy of Science USA* **92**: 295-9.

Xie, J. and McCobb, D. P. (1998). Control of alternative splicing of potassium channels by stress hormones. *Science* **280**: 443-6.

Yanagita, T., Kobayashi, H., Yamamoto, R., Kataoka, H., Yokoo, H., Shiraishi, S., Minami, S., Koono, M. and Wada, A. (2000). Protein kinase C-α and -ε down-regulate cell surface sodium channels via differential mechanisms in adrenal chromaffin cells. *Journal of Neurochemistry* **74**: 1674-84.

Youngson, C., Nurse, C., Yeger, H. and Cutz, E. (1993). Oxygen sensing in airway chemoreceptors. *Nature* **365**: 153-5.

Zhang, J. F., Randall, A. D., Ellinor, P. T., Horne, W. A., Sather, W. A., Tanabe, T., Schwarz, T. L. and Tsien, R. W. (1993). Distinctive pharmacology and kinetics of cloned neuronal Ca^{2+} channels and their possible counterparts in mammalian CNS neurons. *Neuropharmacology* **32**: 1075-88.

Zhu, W. H., Conforti, L., Czyzyk-Krzeska, M. F. and Millhorn, D. E. (1996). Membrane depolarization in PC-12 cells during hypoxia is regulated by an O_2-sensitive K^+ current. *American Journal of Physiology* **271**: C658-65.

THESIS AMMENDMENTS

- Page 83, line 12: the number of perforated patch recordings performed was not given and was, for these experiments, n = 5.

- Page 98, paragraph 2: while high concentrations of cells containing SK channels were observed near venous regions of the adrenal medulla, no data was acquired on the prevalence of these channels in the arteriolar vessels.

- Page 104, line 15: the reference for Pardal and Lopez-Barneo is not given in the reference list and is as follows; Pardal, R. and Lopez-Barneo, J. (2000). Differential effects of mitochondrial inhibitors on the hypoxia sensitivity of glomus cells in slices of rat carotid body. *European Journal of Neuroscience* **12**: 499 (Abstr.). The full manuscript of this abstract has also been recently published; Ortega-Saenz, P., Pardal, R., Garcia-Fernandez, M. and Lopez-Barneo J. (2003). Rotenone selectively occludes sensitivity to hypoxia in rat carotid body glomus cells. *Journal of Physiology* **548**: 789-800.

- Page 145, Figure 5.1: no experiments were carried out using L-, N- and P/Q-type Ca^{2+} channel antagonists simultaneously.

- Chapter 5, Discussion: all experiments were performed on cells cultured for 1-3 days. As culturing may effect the presence of different Ca^{2+} channels in chromaffin cells, it should be noted that the amount which each channel type contributes to the total Ca^{2+} current in the discussed experiments may not necessarily be a direct reflection of the *in vivo* contribution of each channel type.