Glucose-sensitive adrenomedullary neonatal chromaffin cells

The effects of low glucose on catecholamine secretion from neonatal adrenomedullary chromaffin cells

By

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ABSTRACT

Glucose is the primary metabolic fuel in mammalian fetuses, yet mammals are incapable of endogenous glucose production until several hours after birth. Thus, when the maternal supply of glucose ceases at birth there is a transient hypoglycemia in the first few hours of life. This hypoglycemia places the newborn at increased risk of brain damage, especially in newborns of diabetic mothers, premature infants, and newborns small-for-gestational-age. In newborns hypoglycemia evokes a surge in circulating catecholamines and glucagon and a fall in insulin, which triggers the breakdown of glycogen in liver hepatocytes. While this counterregulatory hormonal surge occurs in *adult* mammals the mechanisms of glucose-sensing differ. In adults, neurons in the ventromedial hypothalamus sense glucose and initiate the sympathoadrenal release of catecholamines; however the adrenal gland is not functionally mature until several weeks after birth. Moreover, while glucose acts directly on *adult* pancreatic α cells to elicit glucagon secretion, these cells are glucose-insensitive until several weeks after birth. Thus, I proposed that neonatal adrenomedullary chromaffin cells (AMC) are *direct* glucose-sensors which secrete catecholamines in response to low glucose in the perinatal period.

I used carbon fiber amperometry to measure quantal catecholamine release in response to low glucose (aglycemia and hypoglycemia). Low glucose led to a robust secretory response in a subset of AMCs, however a significant number of AMCs were glucose-insensitive. Despite this fact, these glucose-insensitive AMCs were healthy as evidenced by their robust high K^+-evoked secretion, which was not significantly different than the high K^+-evoked response in glucose-sensitive AMCs. Aglycemia increased the quantal frequency and mean quantal size leading to an increase in catecholamine secretion. On the other hand, hypoglycemia (3 mM glucose) increased quantal frequency and had no effect on quantal size.

I tested for the requirement of voltage-gated Ca^{2+} channel (VGCC) activation in the aglycemia response. The general VGCC blocker Ni^{2+} (2 mM) abolished the aglycemia-evoked secretory response, and moreover, low concentrations of Ni^{2+} (50 μM) blocked the aglycemia-evoked secretory response indicating the involvement of T-type VGCCs. The specific L-type VGCC blocker nifedipine (10 μM) also inhibited the aglycemia-evoked response, indicating that both the L-type and T-type VGCC are necessary for the secretory response during aglycemia.

I developed a fresh thin slice adrenal gland preparation to study the glucose-sensitivity of adrenal glands in slices. This limits phenotypic changes which occur in culture and maintains the integrity of the tissue. In preliminary studies using neonatal adrenal thin slices aglycemia-evoked catecholamine secretion was detected. Aglycemia increased the event frequency but had no effect on quantal size.

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LIST OF ABBREVIATIONS

ADP	*A*denosine *di*phosphate
AMC	*A*dreno*m*edullary *c*hromaffin cell
AMP	*A*denosine *m*ono*p*hosphate
ANOVA	*An*alysis *o*f *va*riance
ATP	*A*denosine *tri*phosphate
BBS	*B*icarbonate-*b*uffered *s*aline
BK	*B*ig conductance calcium-activated K^+ channel
C	*C*oncentration
Ca^{2+}_i	Intracellular calcium
CGA	*C*hromo*g*ranin *A*
CGB	*C*hromo*g*ranin *B*
Chox	*C*hronic *hypox*ia
DAPI	4',6-*dia*midino-2-*p*heny*l*indole
F	*F*araday's constant
fC	*F*emto*c*oulombs
Gluc	*Gluc*ose
Glut	*Gluc*ose *t*ransporter
HCSP	*H*ighly *c*alcium *s*ensitive *p*ool
HIF-2α	*H*ypoxia-*i*nducible *f*actor 2α
IRP	*I*mmediately *r*eleasable *p*ool
K^+_{ATP}	ATP-sensitive K^+ channel
K2p	Two-pore domain K^+ channel
K_{ir}	Inwardly-rectifying K^+ channel
MAH	v-*m*yc infected, *a*drenal derived, *H*NK-1^+

N	*n*umber of moles of catecholamines
n	*n*umber of oxidizable electrons
Nox	*N*orm*ox*ia
pA	*P*ico*a*mp
PBS	*P*hosphate-*b*uffered *s*aline
Q	*Q*uantal Charge
$Q^{1/3}$	Cubed root of quantal charge
r	*R*adius
RLU	*R*elative *l*ight *u*nits
ROS	*R*eactive *o*xygen *s*pecies
SA	*S*ympatho*a*drenal
SgII	*S*ecreto*g*ranin II
SK	*S*mall conductance calcium-activated K^+ channels
TASK	*T*WIK-related *a*cid *s*ensitive K^+ channel
TH	*T*yrosine *h*ydroxylase
TRPC3	*C*anonical *t*ransient *r*eceptor *p*otential channel type *3*
TRPM4	*M*elastatin *t*ransient *r*eceptor *p*otential channel type *4*
V	*V*olume
VGCC	*V*oltage-*g*ated *c*alcium *c*hannel
VMAT	*V*esicular *m*ono*a*mine *t*ransporter

CHAPTER 1

General Introduction

The adrenal glands are paired bilateral endocrine glands which function as one of the primary effector organs of the stress response system in mammals. These highly vascularised organs lie superior to the kidneys and receive arterial blood from the suprarenal arteries and discharge venous blood via the suprarenal veins (Gray, 2005). The glands are innervated by the splanchnic nerve, which is a cholinergic nerve of the sympathetic division of the autonomic nervous system. The adrenal gland is separated into a steroid hormone-producing outer cortex and a catecholamine-producing inner medulla. The cortex is composed of cells which synthesize and release steroid hormones (mineralocorticoids, glucocorticoids, and gonadocorticoids) as part of the hypothalamic-pituitary-adrenal stress axis. The catecholamine-producing cells of the adrenal medulla are round secretory cells called adrenomedullary chromaffin cells (AMCs). These cells, which are sympathetic postganglionic derivatives that have lost their postganglionic axons, synthesize, store, and secrete a number of hormones (i.e., the catecholamines noradrenaline and adrenaline) and other substances into the blood stream during the stress response. A central theme of this thesis is on the ability of these AMCs to act as *direct* metabolic sensors, especially in the perinatal period. There is a particular focus on whether or not these cells can act as low-glucose sensors, a property that could facilitate the critical catecholamine surge necessary for the proper transition of the neonate to extrauterine life. The following background summarizes several aspects of what is currently known about chromaffin cell biology.

Development of chromaffin cells

AMCs arise from a population of multipotent embryonic cells, called neural crest cells, which detach from the dorsal neural tube during embryogenesis (Fernandez-Espejo *et al.*, 2005). During development, sympathoadrenal (SA) progenitor cells migrate ventrally from the apex of the neural tube to the dorsal aorta. During migration, these progenitors differentiate into distinct cell populations including sympathetic neurons, AMCs, carotid body glomus cells, and cells of the organ of Zuckerkandl (Fernandez-Espejo *et al.*, 2005). Several factors are required for SA progenitor cells to differentiate into chromaffin cells; for instance, induction of a catecholaminergic phenotype requires SA progenitor cells to be exposed to bone morphogenetic proteins (Anderson, 1993). Exposure of SA progenitor cells to fibroblast growth factor induces tyrosine hydroxylase expression, the rate-limiting enzyme in catecholamine synthesis. On the other hand, nerve growth factor promotes differentiation of progenitor cells into neurons. Glucocorticoids, supplied via cells in the adrenal cortex, induce the expression of catecholamine synthesizing enzymes in AMCs, and prevent neuronal differentiation. Recently, it has been shown that the transcription factor hypoxia-inducible factor 2α (HIF-2α) is required for the developmental expression of certain catecholamine-synthesizing enzymes (i.e. DOPA decarboxylase and dopamine-β-hydroxylase) in a SA progenitor cell line (Brown *et al.*, 2009).

Organization and contents of chromaffin vesicles

AMCs store transmitters, hormones, and enzymes in membrane bound structures called vesicles. Each AMC contains approximately 10,000 vesicles, constituting 13.5% of the cytoplasmic volume (Crivellato *et al.*, 2008). These vesicles are 150-350 nm in diameter and are known as dense-core vesicles because they appear to have an opaque core when viewed in

electron microscopy. This dense appearance is in part due to the presence of soluble secretory proteins, called granins, in chromaffin vesicles. Three granins are present in chromaffin vesicles; chromogranin A (CGA), chromogranin B (CGB) and secretogranin II (SgII) (Machado *et al.*, 2010). These proteins constitute more than 80% of the soluble vesicular proteins and serve two primary purposes; they act as sorting proteins during vesicle formation, and increase catecholamine storage. Although they share the same principal functions, some differences exist; for instance, CGB has a 100x higher affinity for calcium (Ca^{2+}) than CGA, and is also found in the nucleus where it may regulate transcription (Machado *et al.*, 2010).

Granins contain an N-terminal signal peptide which directs their movement to the endoplasmic reticulum and trans-golgi network, where they are shuttled into vesicles along with other molecules. The low pH and high Ca^{2+} environment of the trans-golgi network causes granins to aggregate with each other and with other vesicular molecules (i.e., catecholamines). Granins form dimers at pH 7.5, however at intravesicular pH (pH = 5.5) they form tetramers (Amatore *et al.*, 2008). Moreover, at pH 5.5 their carboxylic side chains are deprotonated, allowing them to bind to positively charged catecholamines (Machado *et al.*, 2010). These characteristics allow chromogranins to act as a high capacity and low affinity buffer of intravesicular molecules. For instance, CGA binds 32 moles of adrenaline per mol with a K_d of 2.1 mM (Videen *et al.*, 1992). Because the theoretical osmolarity without the effect of chromogranins is >1500 mOsm, chromogranins allow chromaffin vesicles to store high concentrations of catecholamines without swelling (Machado *et al.*, 2010). Indeed, chromaffin vesicles from CGA and CGB knockout mice contain roughly half the concentration of catecholamines compared to controls. Thus granins allow vesicles to store approximately $3x10^6$ catecholamine molecules, corresponding to a concentration of 0.5 M (Wightman *et al.*, 1991).

Catecholamines are synthesized by a series of enzymatic steps occurring in the cytosol and in the chromaffin vesicles. The first step is the rate limiting conversion of L-tyrosine to L-DOPA by the enzyme tyrosine hydroxylase. DOPA decarboxylase converts L-DOPA to dopamine, which is then transported into vesicles. There, it is converted to noradrenaline by the enzyme dopamine β-hydroxylase. Noradrenaline, either stays in the vesicle and is secreted, or it is transported back to the cytosol where is can be converted by phenylethanolamine N-methyltransferase to adrenaline. Adrenaline is then transported back into vesicles to be secreted.

Although adrenaline and noradrenaline are the principal hormones secreted by chromaffin cells, a wide array of other substances are also secreted. These include glycoproteins, prohormone-processing enzymes, protease inhibitors, opioid peptides, peptides, monoamines (i.e., serotonin and dopamine), and nucleotides (i.e., ATP) (García *et al.*, 2006).

Stimulus-secretion coupling in chromaffin cells

In adult mammals catecholamine secretion by AMCs is initiated by splanchnic nerve activation. The splanchnic nerve releases acetylcholine onto AMCs activating nicotinic acetylcholine receptors and provoking membrane depolarization leading to trains of action potentials. This activates several types of voltage-gated Ca^{2+} channels (VGCCs) permitting Ca^{2+} influx across the plasma membrane. A heterogeneous population of VGCCs are present in chromaffin cells including the L-type, N-type, T-type, R-type, and P/Q-type (García *et al.*, 2006). Many factors can change the expression or relative importance of each VGCC type. For instance, in bovine chromaffin cells the population of VGCCs in the whole adrenal gland is different than in cultured chromaffin cells (Benavides *et al.*, 2004). Furthermore, different stimuli may specifically act on different types of VGCCs; for instance the L- and P/Q- types

have been reported to be critical for high K^+-evoked secretion, while the N-type is more important for acetylcholine-evoked secretion (García *et al.*, 2006). Some evidence suggests that the P/Q-type appears to be closer to the secretory machinery than L-type VGCCs, which may have consequences for secretion.

The increase in intracellular Ca^{2+} (Ca^{2+}_i) following stimulation promotes fusion of vesicles with the cell membrane and the release of catecholamines. This process can activate at least two distinct pools of vesicles in AMCs. The immediately releasable pool (IRP) of vesicles is situated near the plasma membrane and these vesicles have a low affinity for Ca^{2+} (García *et al.*, 2006). Single action potentials allow voltage-gated Ca^{2+} entry, creating a microdomain of high Ca^{2+}_i in the vicinity of the plasma membrane. At the mouth of the Ca^{2+} channel this microdomain can reach Ca^{2+}_i concentrations of ~200-300 μM; however this collapses quickly as Ca^{2+}_i is buffered by cytosolic proteins (Berridge, 2010). Vesicles within this microdomain fuse with the plasma membrane and release catecholamines. The second pool of vesicles is a highly Ca^{2+}-sensitive pool (HCSP), located further from Ca^{2+} channels and therefore requiring a larger Ca^{2+}_i load in order to overcome the buffering capacity of the cytosol (García *et al.*, 2006). This pool of vesicles is stimulated by prolonged depolarization and produces asynchronous exocytosis. The expression of different isoforms of the Ca^{2+}-sensitive protein, synaptotagmin, has been proposed to differentiate these two distinct populations of vesicles (Schonn *et al.*, 2008). Two populations of chromaffin vesicles have been reported in mouse AMCs based on the bimodal distribution of vesicular size (Grabner *et al.*, 2005). These authors suggest that small vesicles are more 'fusigenic' and therefore are released during weak stimulation, while robust stimulation leads to the fusion of large vesicles. Whether these two pools of vesicles correspond

to the IRP and the HCSP is unknown. Moreover distinct vesicular pools have been identified due to differences in intracellular location, mobility, and age (Duncan *et al.*, 2003).

Exocytosis of chromaffin vesicles can occur by either full fusion exocytosis or 'kiss-and-run' exocytosis (García *et al.*, 2006). In full fusion exocytosis the membrane of the vesicle fuses with the cell membrane and the entire contents of the vesicle are discharged. During kiss-and-run exocytosis fusion of the vesicle and cell membrane is incomplete, resulting in the formation of only a transient fusion pore. The small diameter of the fusion pore limits the release of catecholamines to soluble species, before the fusion pore is sealed and the vesicle is returned to the cytosol.

Role of the catecholamines in the perinatal mammal

Fetuses are completely dependent on their mothers for the transplacental exchange of oxygen (O_2) and carbon dioxide (CO_2). During birth, this exchange is impaired (e.g. by uterine contractions) leading to a decrease in blood O_2 (hypoxia) and an increase in CO_2/H^+ (acid hypercapnia) in the fetus. These asphyxial stimuli act *directly* on neonatal AMCs and evoke catecholamine release via a non-neurogenic mechanism (Seidler & Slotkin, 1986; Thompson *et al.*, 1997; Munoz-Cabello *et al.*, 2005; Rico *et al.*, 2005). These catecholamines prepare the fetus for air-breathing by increasing fluid absorption and surfactant secretion in the lung, as well as increasing heart contractility (Hagnevik *et al.*, 1983; Sperling *et al.*, 1984). This response can be impaired by various factors, such as maternal nicotine exposure and birth by caesarean section (Irestedt *et al.*, 1984; Slotkin *et al.*, 1995). Impaired catecholamine secretion has been associated with increased risk of sudden infant death syndrome and respiratory distress at birth (Lagercrantz & Slotkin, 1986).

Chemosensing in AMCs

Neonatal AMCs are direct chemosensors that undergo membrane depolarization, voltage-gated Ca^{2+} entry, and secretion of catecholamines in response to asphyxial stimuli as illustrated in Figure 1.1 (Mochizuki-Oda *et al.*, 1997; Thompson *et al.*, 1997; Munoz-Cabello *et al.*, 2005; Rico *et al.*, 2005). Hypoxia-evoked depolarization is facilitated by the closure of large conductance Ca^{2+}-activated (BK) channels, small conductance Ca^{2+}-activated (SK) channels, and delayed rectifier K^+ channels (Thompson & Nurse, 1998). This is offset by the opening of ATP-sensitive K^+ channels (K^+_{ATP}) which favours membrane hyperpolarization (Thompson & Nurse, 1998). On the other hand, hypercapnia activates an inward non-selective cation current as well as inhibits an outward K^+ current leading to membrane depolarization in neonatal AMCs (Munoz-Cabello *et al.*, 2005; Buttigieg *et al.*, 2008).

Hypoxia-, acid-, and hypercapnia-evoked membrane depolarization leads to the entry of extracellular Ca^{2+} through L-type VGCCs (Mochizuki-Oda *et al.*, 1997; Thompson *et al.*, 1997; Munoz-Cabello *et al.*, 2005; Rico *et al.*, 2005). In addition, the T-type and N-type VGCCs appear necessary for the hypoxic response (Mochizuki-Oda *et al.*, 1997; Levitsky & Lopez-Barneo, 2009). Recently, the role of the T-type VGCC has come into question because it was not found in all AMCs and exposure to the specific T-type VGCC inhibitor mibefradil had no effect on hypoxia-evoked catecholamine secretion (Souvannakitti *et al.*, 2010). A second pathway has been proposed to mediate the acid-evoked rise in Ca^{2+}_i which requires Ca^{2+} release from internal stores and extracellular Ca^{2+} (Fujiwara *et al.*, 1994). This rise in Ca^{2+}_i leads to the exocytosis of vesicles containing catecholamines and ATP. During hypoxia, the increase in catecholamine secretion is primarily mediated through an increase in the frequency of quantal events, each of which is thought to arise from exocytosis of a single dense cored vesicle (García-

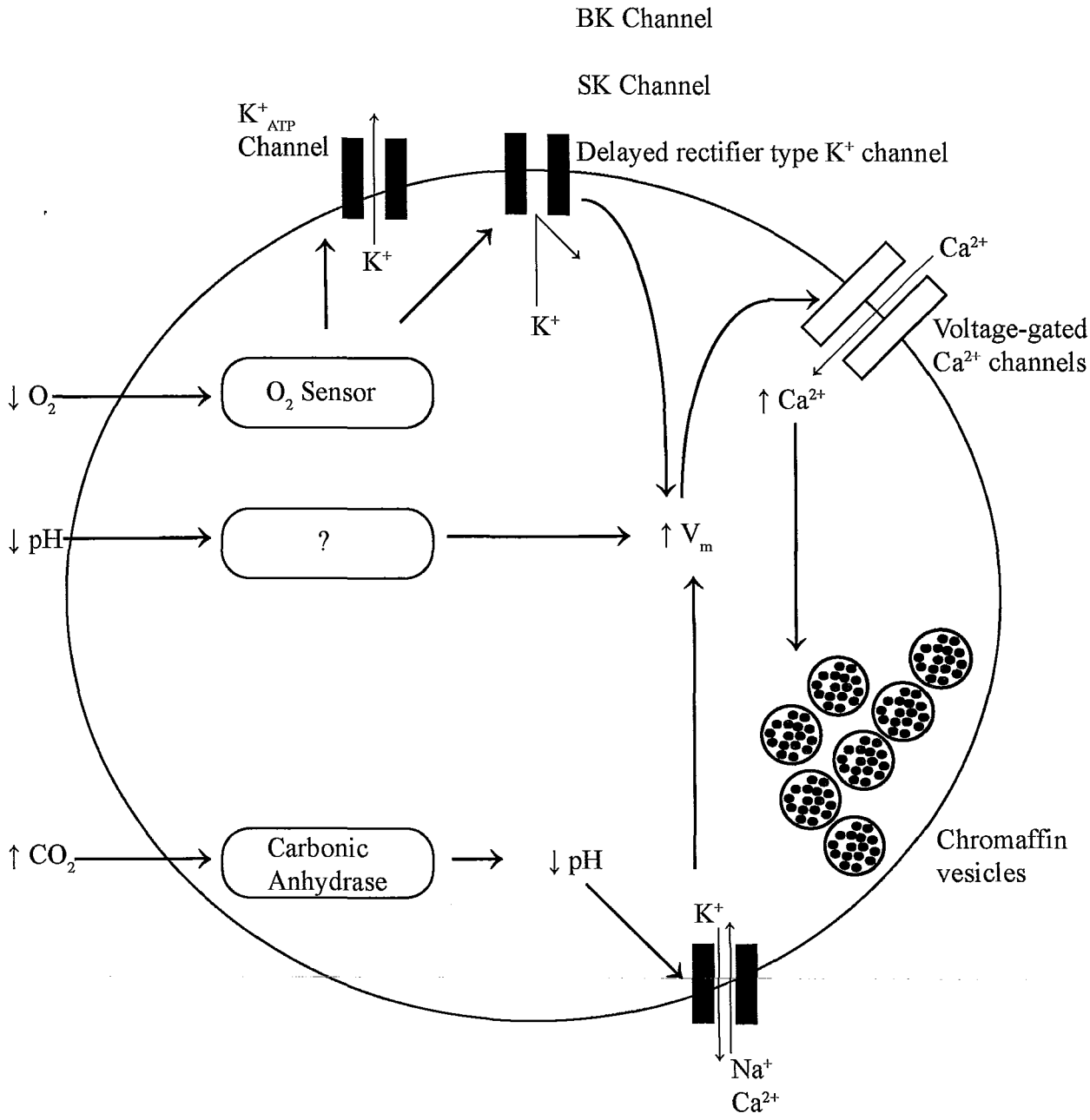
Fernández *et al.*, 2007a). By comparison, hypercapnia appears to increase catecholamine secretion primarily through an increase in mean quantal charge, which is proportional to the number of catecholamine molecules released during each exocytotic event (Munoz-Cabello *et al.*, 2005).

Effects of asphyxia

While sympathoadrenal neurosecretory cells are known to sense independently the asphyxial stimuli discussed above, during asphyxia these stimuli occur concurrently. Therefore it is important to understand how these stimuli interact. Lahiri and Delaney (1975) showed that afferent nerve fibres of carotid body, which contains chemoreceptor glomus cells of sympathoadrenal origin, showed a more-than-additive increase in spike frequency when hypoxia and hypercapnia were applied together. Although there is no conclusive evidence that these stimuli act together at the level of K^+ channel inhibition (Peers, 2004), $Ca^{2+}{}_i$ levels are potentiated, but not in a synergistic manner, when hypoxia and hypercapnia are combined in carotid body glomus cells (Dasso *et al.*, 2000). This indicates that these stimuli synergize at some point during signal transduction.

There are several reports of synergistic effects of hypoxia and hypercapnia on catecholamine release. In whole adrenal gland of the newborn rat, Rico *et al.* (2005) found that acidic hypercapnia and hypoxia together produced a synergistic release of catecholamines. In addition, catecholamine release was greater in chromaffin-derived PC12 cells when hypoxia and acid were applied together than when hypoxia alone was applied (Peers, 2004). These studies indicate that at the cellular level, there may be synergistic relationships between asphyxial stimuli allowing for potentiated neurotransmitter release from chromaffin cells.

Figure 1.1. The chemosensing pathways in neonatal AMCs. Hypoxia activates an O_2 sensor in the cytoplasm of AMCs, which produces an intracellular messenger and decreases ATP. Decrease in ATP opens K^+_{ATP} channels, promoting membrane hyperpolarization. The hypoxia-produced intracellular messenger closes several classes of K^+ channels, leading to membrane depolarization. The pH-sensitive pathway remains unknown, but may involve inhibition of acid-sensitive background K^+ channels (e.g. TASK-1); however, the net result is membrane depolarization. Hypercapnia is proposed to cause intracellular acidification catalyzed by the enzyme carbonic anhydrase, leading to the opening of non-selective cation channels, and consequently, membrane depolarization. Membrane depolarization activates VGCCs, which allows the influx of extracellular Ca^{2+} across the plasma membrane. Increased Ca^{2+}_i promotes fusion of dense core vesicles with the plasma membrane and the release of catecholamines.


BK Channel
SK Channel
K^+_{ATP} Channel
Delayed rectifier type K^+ channel
K^+
K^+
Ca^{2+}
Voltage-gated Ca^{2+} channels
$\downarrow O_2$
O_2 Sensor
$\uparrow Ca^{2+}$
$\downarrow$ pH
?
$\uparrow V_m$
$\uparrow CO_2$
Carbonic Anhydrase
$\downarrow$ pH
Chromaffin vesicles
K^+
Na^+
Ca^{2+}

Developmental regulation of chemosensing

Hypoxia directly evokes catecholamine secretion from AMCs of perinatal rats, however this response is suppressed postnatally, coincident with the maturation of splanchnic innervation after the first postnatal week (Seidler & Slotkin, 1985). Moreover, in juvenile rats (> 2 weeks postnatal) denervation of the splanchnic nerve partially restores the direct hypoxia-evoked catecholamine release. Two theories have been proposed to account for this loss of chemosensitivity: (1) that *chronic* exposure to neurotransmitters released from the splanchnic nerve induces changes in gene expression that render AMCs no longer chemosensitive, and/or (2) that neurotransmitters *acutely* inhibit the channels that cause membrane depolarization.

Several lines of evidence suggest that most juvenile AMCs are unable to sense asphyxial stimuli. For instance, the majority of rat AMCs from postnatal day 1-3 respond to hypoxia after short term culture, however this response is absent in juvenile AMCs (Thompson *et al.*, 1997). Likewise, in thin adrenal slices, both the proportion of hypoxia- and hypercapnia-sensitive cells and the magnitude of the responses are significantly less in juveniles than in neonates (Munoz-Cabello *et al.*, 2005; García-Fernández *et al.*, 2007a). Similarly, in whole adrenal glands, acid-evoked catecholamine secretion in juveniles is less than half the secretion measured in neonates (Rico *et al.*, 2005).

This loss of chemosensing has been proposed to be mediated through chronic stimulation of the nicotinic acetylcholine receptor. Nicotine exposure *in utero* and *in vitro* abolishes the hypoxic response via upregulation of the K^+_{ATP} channel (Buttigieg *et al.*, 2008; Buttigieg *et al.*, 2009). Thus, during hypoxia more K^+_{ATP} channels will open and favour membrane hyperpolarization. However, in animals subjected to nicotine exposure *in utero*, the response to hypercapnia remains intact, suggesting that other mechanisms appear necessary to explain the

loss of hypercapnic chemosensitivity. Loss of hypoxia-sensing has also been proposed to be mediated by the developmental regulation of T-type VGCC (Levitsky & Lopez-Barneo, 2009). These channels are present in neonatal AMC where they appear necessary for the hypoxic response, however their expression decreases with age (Levitsky & Lopez-Barneo, 2009).

In contrast, there are several reports of hypoxia-sensing in *adult* rat chromaffin cells, where hypoxia leads to inhibition of K^+ currents, a rise in Ca^{2+}_i, and catecholamine release (Mochizuki-Oda *et al.*, 1997; Lee *et al.*, 2000). Indeed, these studies reported that approximately 50% of *adult* chromaffin cells were hypoxia-sensitive with inhibition of the K^+ current and a rise in Ca^{2+}_i. Further, acute neonatal and adult slices responded with similar increases in Ca^{2+}_i in P5, P11, and 8 week old rats and this was not abolished by cholinergic blockers (Takeuchi *et al.*, 2001). In addition, hypoxia leads to depolarization in adult guinea pig AMCs through activation of a cation conductance (Inoue *et al.*, 1998).

Keating *et al.* (2004) have proposed that cultures of juvenile ovine AMCs are O_2-sensitive, and that the *acute* release of opioids from the splanchnic nerve blocks hypoxia-sensing. In these AMCs, combined μ- and κ-opioid agonists blocked the hypoxia-evoked rise in Ca^{2+}_i and catecholamine secretion. This effect occurs through μ-receptor mediated enhancement of current through SK channels, and a κ-receptor mediated decrease in the conductance of BK channels. However, acute opioid receptor stimulation failed to prevent acid-sensing by isolated rat neonatal adrenal glands (Rico *et al.*, 2005). Thus, there may be species and/or stimulus-specific mechanisms which account for the loss of chemosensing in perinatal AMC.

Evidence for AMCs as glucosensors

In addition to O_2 and CO_2, fetuses are completely dependent on their mothers for the transplacental facilitated diffusion of glucose. Although glucose is the primary metabolic fuel in the fetus, glycogenolysis and gluconeogenesis are absent *in utero* (Hay *et al.*, 1981). During normal birth this transport ceases, leading to a decrease in glucose (hypoglycemia) in the first few hours of life (Hagnevik *et al.*, 1983; Irestedt *et al.*, 1984; Srinivasan *et al.*, 1986; Tanzer *et al.*, 1997; Marom *et al.*, 2010). In the newborn hypoglycemia activates a counterregulatory surge in catecholamines and glucagon, and a fall in insulin (Cuezva *et al.*, 1982). These hormonal changes stabilize blood glucose by initiating glycogenolysis in liver hepatocytes (Hunter, 1969; Girard *et al.*, 1973b). Despite this response, over 2% of newborns experience hypoglycemia and require clinical intervention (DePuy *et al.*, 2009), increasing the risk of brain damage (Lucas *et al.*, 1988). Moreover, premature infants, newborns of diabetic mothers, and neonates small-for-gestational age are at increased risk of hypoglycemia, which is one of the leading causes of increased mortality and morbidity among these individuals (Williams, 1997).

In adult mammals, hypoglycemia acts via hypothalamic neurons to initiate the neurogenic release of catecholamines from AMCs, and on pancreatic α and β cells to evoke glucagon secretion and inhibit insulin release. However, pancreatic α cells are glucose-insensitive until several weeks after birth (Edwards *et al.*, 1972; Lernmark & Wenngren, 1972; Marliss *et al.*, 1973; Sodoyez-Goffaux *et al.*, 1979), and splanchnic innervation of the adrenal gland is immature in most neonatal mammals (including rats and humans) (Artal, 1980; Tomlinson & Coupland, 1990). In newborn rats, adrenalectomy or infusion with adrenergic antagonists prevents hepatic glycogen breakdown and causes severe hypoglycemia (Girard *et al.*, 1973a). It has recently been demonstrated that aglycemia produces membrane depolarization and a rise in

Ca^{2+}_i in a subset of immortalized AMCs derived from embryonic day 14 fetal rat adrenal medulla (Piskuric *et al.*, 2008). These data suggest that counterregulatory changes in response to hypoglycemia require catecholamine secretion via a non-neurogenic mechanism in the newborn.

There is conflicting evidence of the effects of hypoglycemia on catecholamine secretion from *adult* AMCs. In adult rats, prolonged hypoglycemia (<4 mM) produced a dramatic increase in circulating catecholamines, and this effect was abolished by adrenalectomy but not by adrenal denervation (Khalil *et al.*, 1986). On the other hand, in isolated adult rat adrenal glands, hypoglycemia had no effect on catecholamine secretion (de Araujo *et al.*, 1999).

Mechanisms of glucosensing in other cell types

Direct glucose-sensing cells have been reported in the hypothalamus, the hepatic portal vein, the pancreas, the nodose ganglia, brain astrocytes, and the carotid body (Burcelin *et al.*, 2000; Pardal & Lopez-Barneo, 2002; Kang *et al.*, 2004; Quesada *et al.*, 2008). In most cells, the major regulatory step in glucose metabolism involves either the transport of glucose across the plasma membrane or the metabolism of glucose inside the cell. Following metabolism, an unknown metabolite is proposed to evoke membrane depolarization in these cells; however, the ion channels involved seem to be distinct for each cell type. Membrane depolarization leads to voltage-gated Ca^{2+} entry, followed by the release of neurotransmitters or hormones.

Glucose transport across the plasma membrane is facilitated by a family of glucose transporters (Glut). Glut2, the high flux transporter, facilitates glucose uptake by pancreatic β cells and is required for insulin secretion (Thorens, 2003). However, the importance of glucose transport for hypoglycemia-sensing cells is less clear. Mice lacking the Glut2 gene have

impaired hypoglycemia-induced glucagon secretion and impaired hepatoportal vein glucosensing, even though α cells of the pancreas do not express Glut2 (Burcelin *et al.*, 2000; Thorens, 2003). This suggests that the knockout of Glut2 affects α cells via some indirect mechanism. Moreover, Glut2 is ubiquitously expressed in the hypothalamus; however, Glut3 and Glut4 are the transporters present in glucosensing neurons (Kang *et al.*, 2004). Furthermore, Glut1, 3, and 4 are expressed in the carotid body, however Glut2 is not (García-Fernández *et al.*, 2007b). This heterogeneous population of glucose transporters suggests that a specialized transport process is not a critical step for glucosensing.

In contrast, there is growing evidence that the metabolism of glucose regulates glucosensing. In pancreatic α cells and dorsal vagal neurons inhibitors of cellular metabolism mimic the effect of aglycemia on membrane potential and secretion (Balfour & Trapp, 2007; Gromada *et al.*, 2007; Grabauskas *et al.*, 2009). Inhibition of glucose metabolism by the hexokinase inhibitor 2-deoxyglucose leads to catecholamine secretion in carotid body glomus cells (García-Fernández *et al.*, 2007b). However, dialyzing cells with high intracellular ATP does not block the aglycemia-induced inhibition of outward current (Pardal & Lopez-Barneo, 2002). Indeed, this is the pattern observed in hypothalamic orexin neurons, where glucose acts extracellularly to induce membrane depolarization (Burdakov *et al.*, 2006). In these neurons some intermediary of glucose metabolism probably acts as a second messenger, but this is neither ATP nor Ca^{2+}_i. Moreover, in glucosensing cells of the hepatoportal vein, lactate or pyruvate abolishes the hypoglycemic response (Matveyenko & Donovan, 2006). In contrast, in the ventromedial hypothalamus and in orexin neurons lactate potentiates the increase in action potential frequency induced by hypoglycemia (Song & Routh, 2005; Gonzalez *et al.*, 2009).

The phosphorylation of glucose by hexokinase IV (glucokinase) appears to be the major regulatory step in cells which require glucose metabolism. Glucokinase is the rate-limiting enzyme in glucose metabolism, and therefore probably glucosensing; it catalyzes the phosphorylation of glucose into glucose-6-phosphate (Quesada *et al.*, 2008). It is the lowest affinity hexokinase, and the only one without known feedback inhibition. Pharmacological inhibition of glucokinase potentiates the hypoglycemia-induced rise in $Ca^{2+}{}_i$ by lowering the ATP/ADP ratio (Dunn-Meynell *et al.*, 2002). Furthermore, the addition of a glucokinase activator leads to a decrease in $Ca^{2+}{}_i$ oscillations in hypoglycemia-sensing neurons (Kang *et al.*, 2006). Knockdown of glucokinase results in an almost complete loss of hippocampal glucosensing neurons (Kang *et al.*, 2006). Glucokinase appears to be expressed in pancreatic α cells and glucosensing cells of the hepatoportal vein; however its physiological importance remains to be elucidated (Heimberg *et al.*, 1996; Burcelin *et al.*, 2000). Further, single-cell RT-PCR revealed that glucokinase is expressed in only 43% of hippocampal hypoglycemia-sensing neurons (Kang *et al.*, 2004). Moreover, it is not expressed in carotid body glomus cells, and inhibition of glucokinase has no effect on orexin neurons (García-Fernández *et al.*, 2007b; Gonzlez *et al.*, 2008). Thus, glucokinase appears to be important in some cell types, but there must be other mechanisms involved in the regulation of glucosensing.

In general, metabolism of glucose into ATP is a necessary step leading to membrane depolarization. In pancreatic α cells, hypoglycemia decreases ATP leading to the opening of the $K^+{}_{ATP}$ channel (Quesada *et al.*, 2008). Opening of this channel hyperpolarizes the cell to a membrane potential which removes inactivation of T-type VGCC, leading to Ca^{2+} influx and depolarization of the cell towards threshold. At this point voltage-gated Na^+ channels open leading to regenerative action potentials. In α cells hyperglycemia closes the $K^+{}_{ATP}$ channel

depolarizing the membrane; however no action potentials are produced due to the inactivation of the majority of the voltage-gated Na^+ channels. In contrast, hyperglycemia closes K^+_{ATP} channels in pancreatic β cells leading to membrane depolarization and action potential firing (Thorens, 2003). In rat dorsal vagal neurons hypoglycemia decreases ATP, leading to inhibition of the Na^+-K^+-ATPase combined with closure of a background K^+ channel with TASK-like properties (Balfour & Trapp, 2007). In arcuate nucleus neurons, hypoglycemia closes the cystic fibrosis transmembrane conductance regulator, a Cl^- channel, leading to membrane depolarization (Fioramonti *et al.*, 2007). Long periods (~10 minutes) of aglycemia leads to the development of a mixed Na^+/K^+ current in striatal spiny neurons (Calabresi *et al.*, 1997). Glucose acts extracellularly through an unknown second messenger to close a two-pore-domain K^+ channel (K2p) in orexin neurons (Burdakov *et al.*, 2006). However, because orexin neurons in K2p knockout mice are still glucosensitive, closure of the inward rectifying K_{ir} or SK channels have been proposed to mediate glucosensing (Guyon *et al.*, 2009). Carotid body glomus cells, which are developmentally related and functionally similar to AMCs, respond to hypoglycemia by closure of a K^+ channel along with opening of a TRP-like channel, possibly of the TRPC3 subtype (García-Fernández *et al.*, 2007b). In another study, hypoglycemia was found to produce membrane depolarization by activation of a TRPM4 channel in a subpopulation of brain astrocytes (Chen & Simard, 2001). This channel is coupled to the ATP-sensitive sulfonylurea receptor subunit rendering the cation channel ATP-sensitive (Chen & Simard, 2001).

Carbon Fiber Amperometry

The fusion of a vesicle with the cell membrane and extrusion of its contents is known as exocytosis. Carbon fiber amperometry was developed to measure the exocytosis of single

vesicles of oxidizable material (Wightman *et al.*, 1991). In amperometry, a carbon fiber microelectrode is held at a constant potential sufficient to oxidize the molecule of interest and the current is measured over time. Carbon fiber electrodes typically have a radius of 5 μm, which is small enough to measure exocytosis from a single chromaffin cell (diameter: ~15 μm).

Exocytosis proceeds by several steps identified using the patch-amperometry technique. In this technique a carbon fibre microelectrode is used to measure catecholamine secretion from a single patch-clamped chromaffin cell whereas the patch clamp recording is used to measure changes in cell capacitance, which is proportional to surface area. When vesicles fuse with the plasma membrane, the capacitance increases, and catecholamines can be measured as a current at the carbon fibre microelectrode. The first detectable step is the movement of the neurohormone or transmitter through a transient fusion pore. The fusion pore is an aqueous channel created by the initial fusion of the vesicle with the plasma membrane. Neurohormones that are not bound to granins in the vesicular matrix can move through this channel and be detected; this signal is known as a "foot". In "kiss-and-run" exocytosis, the vesicle then retracts from the cell membrane and transmitter release ceases. In full fusion exocytosis there is a sharp increase in the current to the peak amplitude. This is due to opening of the fusion pore and an increase in free catecholamines as they lose their affinity for granins. There is then a slow decay back to baseline. These current spikes have been observed in many different cell types, including chromaffin cells, mast cells, β-cells, and neurons.

The area under each current spike gives the quantal charge (Q), and the relationship between quantal charge and number of catecholamine molecules per vesicle is given by the equation:

$$Q = nFN$$

Where n is the number of oxidizable electrons (2 per catecholamine molecule), F is Faraday's constant, and N is the number of moles of catecholamines. The number of moles of catecholamines is a product of the concentration of catecholamines in a vesicle (C) and the vesicular volume (V), therefore:

$$Q = nFCV$$

If it is assumed that the concentration of catecholamines is constant, then:

$$Q \propto V$$

Since vesicles are approximately spherical, the volume is given by:

$$V = \frac{4}{3}\pi r^3$$

Therefore:

$$Q \propto \frac{4}{3}\pi r^3$$

$$Q \propto r^3$$

$$Q^{\frac{1}{3}} \propto r$$

Thus, $Q^{1/3}$ is proportional to vesicular radius when the concentration of catecholamines is constant. This may be a good assumption, given the distribution of vesicular radii calculated with this method is similar to the distribution measured by electron microscopy (Finnegan *et al.*, 1996). Moreover, this method shifts the distribution curve for quantal charge from a right-skewed distribution to a Gaussian distribution which allows for easier statistical analysis.

Several experimental factors are important in measuring catecholamines from vesicles during fusion. Catecholamines are mostly bound to chromogranins, and vesicles have a very high concentration of soluble molecules, much higher than the isotonic extracellular solution (~315 mOsM). Reducing this osmotic gradient through a hypertonic solution (630 mOsm)

reduces current spikes and decreases the number of catecholamine molecules secreted (Borges *et al.*, 1997). On the other hand, hypotonic solutions increase the mean quantal charge. The intravesicular pH is approximately 5.5, which creates a large proton gradient relative to the extracellular face (pH = 7.4). This pH gradient is critical in catecholamine release, as the affinity of granins for catecholamines is pH-dependent. Increasing extracellular pH increases the proton gradient resulting in a larger quantal charge. In addition, other factors such as temperature, ionic composition, and time in culture can affect quantal charge (Jankowski *et al.*, 1992; von Ruden & Neher, 1993; Pihel *et al.*, 1994; Tang *et al.*, 2005).

Organization of this thesis

The primary hypothesis of this thesis is that newborn rat AMCs respond to a decrease in glucose (hypoglycemia or aglycemia) via voltage-gated Ca^{2+} entry and catecholamine secretion. A general introduction to this topic and rationale for this hypothesis is presented in Chapter 1. The principal model used in these experiments was an *in vitro* culture preparation of primary newborn rat AMCs. Carbon fiber amperometry allowed for the detection of catecholamine release from cells in culture. Furthermore, the use of different VGCCs blockers enabled me to probe for the contributions of different VGCCs subtypes in the aglycemia response. The results from these experiments are presented in Chapter 2. In Chapter 3, I present my results examining the effects of chronic hypoxia on aglycemia-evoked secretion. Chemosensory responses from rat AMCs proved difficult measure in an *in vitro* model; some of the difficulties and attempted solutions are presented in Appendix I. In response to these difficulties, an adrenal gland slice preparation was developed thereby avoiding the enzymatic treatments required for cultured cells; the preliminary results from this project are presented in Appendix II. Since ATP is co-stored

along with catecholamines in chromaffin vesicles, I attempted to measure ATP secretion is dispersed neonatal AMCs, juvenile adrenal slices, and whole neonatal adrenal glands. My attempts at measuring ATP secretion are presented in Appendix III. The formula for the medium used in these studies is presented in Appendix IV.

CHAPTER 2

Neonatal adrenomedullary chromaffin cells secrete catecholamines in response to low glucose

SUMMARY

The effects of low glucose on catecholamine secretion from neonatal adrenomedullary chromaffin cells (AMCs) were investigated using carbon fiber amperometry. Low glucose evoked a robust secretory response from a subset of neonatal AMCs resulting in increased catecholamine secretion. Aglycemia (0 mM glucose) increased both the frequency of quantal vesicular release and mean quantal charge. In contrast, mild hypoglycemia increased the frequency but had little effect on quantal charge. In addition to these glucose-sensitive AMCs, there was a population of glucose-insensitive AMCs which showed no change in catecholamine secretion in response to aglycemia. These glucose-insensitive AMCs appeared healthy as demonstrated by their robust high K^+-evoked secretion, which was similar to that seen in glucose-sensitive AMCs.

The requirement for voltage-gated Ca^{2+} channel (VGCC) activation during the aglycemia-evoked secretory response was also tested. The general VGCC blocker Ni^{2+} (2 mM) abolished this secretory response. The fact that even low concentrations of Ni^{2+} (50 μM) also inhibited the aglycemia-evoked secretory response suggested the involvement of T-type VGCC. Moreover, the specific L-type VGCC blocker nifedipine (10 μM) was also effective in inhibiting the aglycemia-evoked secretory response, suggesting that the overall aglycemia-evoked catecholamine secretion in neonatal AMC requires the influx of Ca^{2+} through both T-type and L-type VGCCs.

Because newborns are often exposed to hypoxia and low glucose in concert, for example during the asphyxia associated with uterine contractions at birth, and given that each stimulus could separately evoke catecholamine secretion, the combined effect of these two stimuli on secretion was investigated. Together, hypoxia and aglycemia caused an increased secretory response when compared with the effect of either stimulus in isolation, though it was unclear whether this was attributable to an increased frequency of quantal events. Taken together, these results suggest that both hypoxia and aglycemia are key contributors to the critical catecholamine surge required for the proper transition of the neonate to extrauterine life.

INTRODUCTION

Occlusion of the umbilical cord at birth prevents the transfer of O_2 and CO_2, producing fetal hypoxia and hypercapnia. This leads to a robust increase in circulating catecholamines which prepares the fetus for life outside the womb by increasing heart contractility, initiating lung fluid absorption, and activating lung surfactant secretion (Lagercrantz & Slotkin, 1986). In addition to O_2 and CO_2, glucose transport from the mother to the fetus is terminated at birth. This is critical since glucose is the primary metabolic fuel of the fetus, and fetuses are incapable of producing glucose by glycogenolysis or gluconeogenesis (Hay *et al.*, 1981). This results in a severe hypoglycemia in the first few hours of life (Hagnevik *et al.*, 1983; Irestedt *et al.*, 1984; Srinivasan *et al.*, 1986; Tanzer *et al.*, 1997; Marom *et al.*, 2010). In the newborn, hypoglycemia activates a counterregulatory surge in catecholamines which stabilizes blood glucose by activating hepatic glycogen breakdown (Hunter, 1969; Girard *et al.*, 1973b; Cuezva *et al.*, 1982).

Hypoxia and hypercapnia act *directly* on neonatal AMCs to evoke membrane depolarization, voltage-gated Ca^{2+} entry, and catecholamine secretion (Mochizuki-Oda *et al.*, 1997; Thompson *et al.*, 1997; Munoz-Cabello *et al.*, 2005). Moreover, this property is

developmentally regulated and is lost along a time course similar to maturation of splanchnic innervation of the adrenal gland (Seidler & Slotkin, 1986). Recently, a subset of immortalized embryonic AMCs were shown to undergo membrane depolarization and a rise in Ca^{2+}_i in response to aglycemia (Piskuric *et al.*, 2008). In this study I tested the hypothesis that neonatal AMCs respond to low glucose via voltage-gated Ca^{2+} entry and catecholamine secretion.

MATERIALS AND METHODS

Animal Procedures

Pregnant Wistar rats (Charles River, Quebec, Canada) were delivered to our animal care facility and gave birth naturally two weeks later. They were given *ad libitum* access to food and water and kept under a 12 h light-dark cycle at 22°C. Newborn rats (0-2 days old) were rendered unconscious by a blow to the back of the head and then killed by decapitation before removal of the adrenal glands. All procedures and experiments were approved by the Animal Research and Ethics Board at McMaster University, according to the Canadian Council for Animal Care guidelines.

Cell Culture Method

Adrenal glands were removed from postnatal day 0 (P0) rats and placed in L-15 plating medium (See Appendix IV). Most of the surrounding cortical tissue was removed and discarded, and the remaining medullary regions were incubated for 1 h at 37°C in an enzymatic solution containing 0.1% trypsin (Gibco), 0.1% collagenase (Gibco), and 0.01% deoxyribonuclease (Millipore). Following digestion, the enzymatic solution was removed and replaced with a modified F-12 medium (GIBCO) supplemented with 5% fetal calf serum (GIBCO), 80 U/l insulin (Sigma), 0.6% glucose, 1% penicillin and streptomycin (GIBCO), and 0.01%

dexamethasone (Sigma). The medullary tissue was then mechanically dissociated using forceps, triturated with a Pasteur pipette, and the dispersed cells pelleted by centrifugation. Cells were then plated into culture dishes in modified F12 medium. They were grown at 37°C in an incubator with a humidified atmosphere of 95% air/5% CO_2 for 1-2 days before experiments were performed.

Immunofluorescence

Cultured AMCs were grown on a glass coverslip attached to a modified cell culture dish. Cultures were rinsed in pre-warmed (37°C) phosphate-buffered saline (PBS) before fixation for 1 hour at -20°C in 95% methanol/5% acetic acid. Cells were then washed 3 times for 3 minutes each in PBS, before incubation overnight with polyclonal tyrosine hydroxylase (TH) antibody (rabbit, 1:2000 dilution; Chemicon, El Segundo, CA, USA) at 4°C. They were then washed 3 times for 10 minutes each in PBS then incubated in FITC-conjugated secondary antibody (goat anti-rabbit IgG, 1:50 dilution; Cappel, Malvern, PA, USA) for 1 hour in the dark. Cells were washed 3 times for 3 minutes each in PBS, and then Vectashield (Vector Laboratories, Burlingame, CA) was added to prevent photobleaching. Immunostaining was visualized with a Zeiss (IM35) inverted microscope equipped with epifluorescence.

Carbon Fiber Amperometry

Cell cultures were placed on the stage of an inverted microscope (Accu-Scope 3030) and perfused under gravity with a HEPES-buffered recording solution at pH 7.4 (see below). Catecholamine secretion was monitored by placing a ProCFE carbon fiber electrode (Dagan) in close apposition to the plasma membrane of an AMC. The electrode was connected to a CV

203BU headstage and an Axopatch 200B amplifier set at +800 mV. Data were filtered at 100 Hz and digitized at 250 Hz using Clampex 9.2 (Axon Instruments) and subsequently analyzed with Clampfit 9.2 (Axon Instruments). Individual secretory events were rejected if they were below 2.58 times the standard deviation (99% confidence interval) of the baseline noise (typically 1-3 pA) and did not have an exponential decay. The quantal charge was measured as the integrated area under each spike, and the mean quantal charge per cell was used for analysis (Colliver *et al.*, 2000). The $Q^{1/3}$ was calculated by taking the cube root of quantal charge. Frequency was calculated as the number of single quantal events per minute and secretion rate was measured as the cumulative charge resulting from the sum of successive quantal events over a given time interval. AMCs were considered glucosensitive if there was a >2 fold increase in secretion rate when cells were exposed to aglycemia (i.e. 0 mM glucose) compared to control (5 mM glucose). Furthermore, only healthy cells, i.e. those which responded to high K^+ (30 mM KCl) with robust catecholamine secretion were analyzed for responses to low glucose.

Solutions

HEPES-buffered saline contained (mM): NaCl, 135; HEPES, 10; glucose, 0-10; sucrose, 10-0; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 2 at pH= 7.4. The concentration of glucose ranged from 10 mM to 0 mM and the osmolarity was kept constant by equimolar substitution with sucrose. Solutions were made hypoxic (PO_2 ~15 mmHg) by bubbling HEPES-buffered saline with 100% N_2 gas. High K^+ (30 mM K^+) solutions were kept at constant osmolarity by equimolar substitution with NaCl. Nickel and nifedipine were purchased from Sigma and 1000x stock solutions were made fresh before each experiment.

Statistical Analysis

Results are expressed as the mean ± S.E.M. Statistical comparisons were performed with Student t-tests or ANOVA where appropriate.

RESULTS

Aglycemia-evoked changes in catecholamine secretion

When the perfusion solution was changed from one containing 5 mM glucose to another with 0 mM glucose there was a robust secretory response from some neonatal AMCs (Fig 2.1A, middle). The reversible bursts of spike-like activity are qualitatively similar to the secretory responses evoked by hypoxia and hypercapnia in previous studies on neonatal AMCs (Munoz-Cabello *et al.*, 2005; García-Fernández *et al.*, 2007a). Each spike represents the release of a single secretory vesicle (i.e. a quantum) containing catecholamines (See Fig 2.1, lower). The fast rise time and slow exponential decay of these amperometric spikes is characteristic of vesicular catecholamine release (Wightman *et al.*, 1991). The large increase in cumulative charge during aglycemia was indicative of a robust catecholamine secretion induced by this stimulus (Fig 2.1, upper). Cultured neonatal AMCs stained positively for TH, the rate limiting enzyme in catecholamine synthesis, thus confirming the catecholaminergic phenotype of these cells (Fig 2.1B)

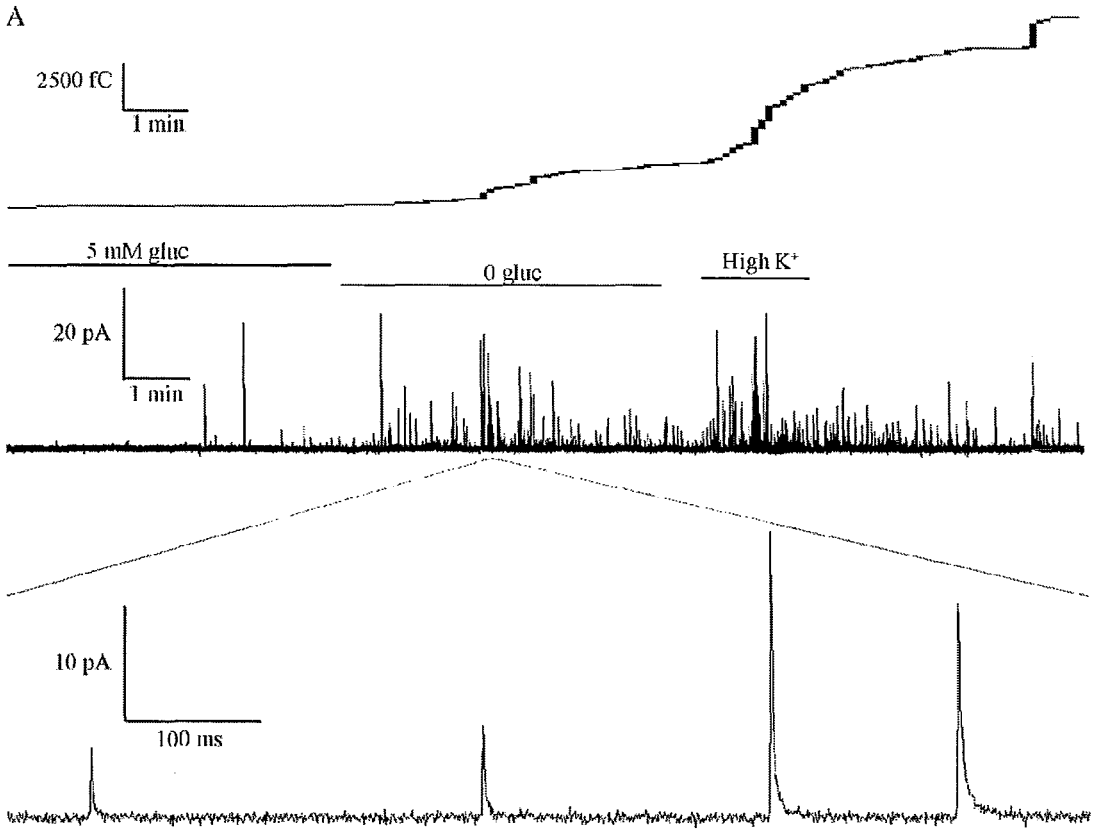
The increased catecholamine secretion during aglycemia could be attributable to an increase in frequency of exocytotic events, mean quantal charge, or both. Aglycemia increased the frequency of quantal events ~3.4 fold (Fig 2.2A) ($p<0.001$, n = 17 cells), and increased mean quantal charge ~2.7 fold (Fig 2.2B) ($p<0.001$, n = 17 cells). $Q^{1/3}$, a measure of vesicular radius, increased ~1.4 fold in 0 mM glucose compared to 5 mM glucose (Fig 2.2D) ($p<0.001$, n =17 cells). In responsive cells, the secretion rate, estimated from the product of mean quantal charge

and event frequency, increased from 98.6 ± 31.6 fC/min during 5 mM glucose to 789.8 ± 236.5 fC/min during aglycemia (Fig 2.2C) ($p<0.01$, n = 17 cells).

Some cells showed no secretory response to aglycemia, but otherwise appeared healthy based on the presence of a robust high K^+-evoked secretion (Fig 2.3A). In these cells exposure to 0 mM glucose did not result in a significant change in the frequency of quantal events (Fig 2.3A) ($p>0.30$, n =33 cells) or quantal charge (Fig 2.3C) ($p>0.96$, n = 33 cells). For this group, the basal secretion rate was 74.3 ± 14.6 fC/min in normal glucose (5 mM) compared to 73.8 ± 10.7 fC/min in 0 mM glucose (Fig 2.3D) ($p>0.88$, n = 33 cells). Moreover, there was also no change in $Q^{1/3}$ in response to aglycemia in these cells (Fig 2.3E) ($p>0.80$, n = 33 cells).

To determine whether cells non-responsive to aglycemia (i.e. glucose-insensitive) were otherwise healthy, or at least were capable of generating Ca^{2+}-mediated secretory responses, the effects of the depolarizing stimulus high K^+ were tested. Both the glucose-sensitive and glucose-insensitive populations of AMC discussed above responded to high K^+ with robust catecholamine secretion. There was no statistically significant difference in event frequency (Fig 2.4A), or mean quantal charge (Fig. 2.4B), between these two populations of cells. As a result, there was no significant difference in secretion rate (Fig 2.4C), and furthermore, there was also no change in $Q^{1/3}$ (Fig 2.4D). Thus, the glucose-insensitive neonatal AMCs analysed in this study had intact mechanisms for stimulus-secretion coupling, but could have been missing a vital component (e.g. protein) required for low glucose-sensing. It should be noted that in the course of this study many cells (> 50) were encountered that failed to respond to high K^+, but appeared normal under phase contrast microscopy. These cells were considered unhealthy and excluded from further analysis.

Figure 2.1. Aglycemia-evoked catecholamine release from a neonatal AMC. A, *upper*:, cumulative charge of catecholamine release corresponding to the cell below; *middle*: example of a robust secretory response in a single AMC in response to aglycemia; cell was exposed sequentially to control solution (5 mM glucose), aglycemia (0 mM glucose), and high K^+ (30 mM KCl) during the period indicated by upper horizontal bars; *lower*: expanded time scale trace of amperometric (current) spikes corresponding to the indicated time period during aglycemia; each spike represents the fusion of a single secretory vesicle. B, positive immunofluorescence staining of single and small clusters of neonatal AMCs for tyrosine hydroxylase (TH).


A
2500 fC
1 min
5 mM gluc
0 gluc
High K+
20 pA
1 min
10 pA
100 ms

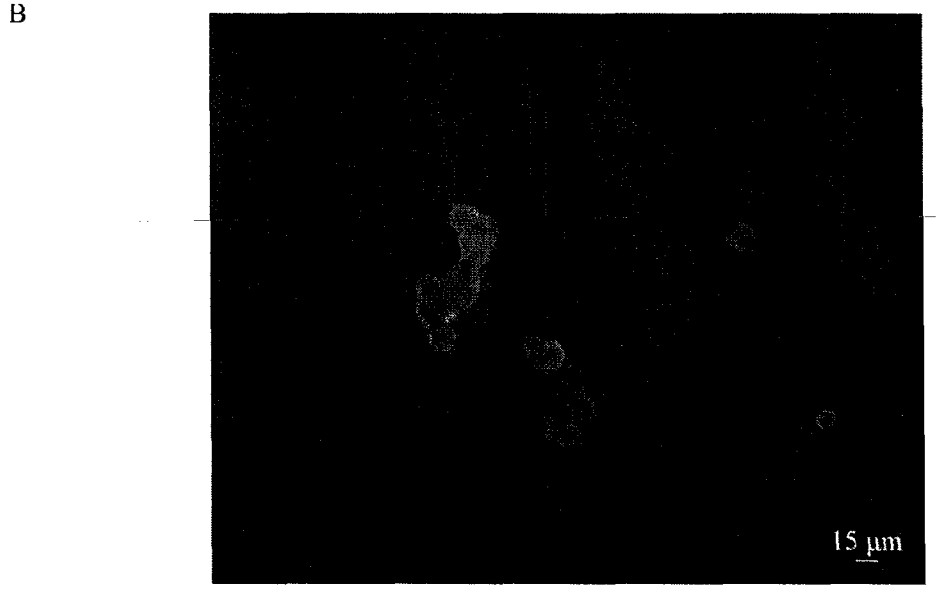

B
15 µm

Figure 2.2. Quantification of aglycemia-evoked catecholamine secretion from cultured neonatal rat AMCs. A, aglycemia increased the frequency of single exocytotic events compared to normoglycemia (5 mM glucose). B, quantal charge increased during aglycemia. C, secretion rate, estimated from the product of mean quantal charge and frequency, increased during aglycemia. D, $Q^{1/3}$, a measure of vesicular volume, increased during aglycemia. Data were compared with a two-way Student's t-test (**$p<0.01$, ***$p<0.001$, $n = 17$ cells).

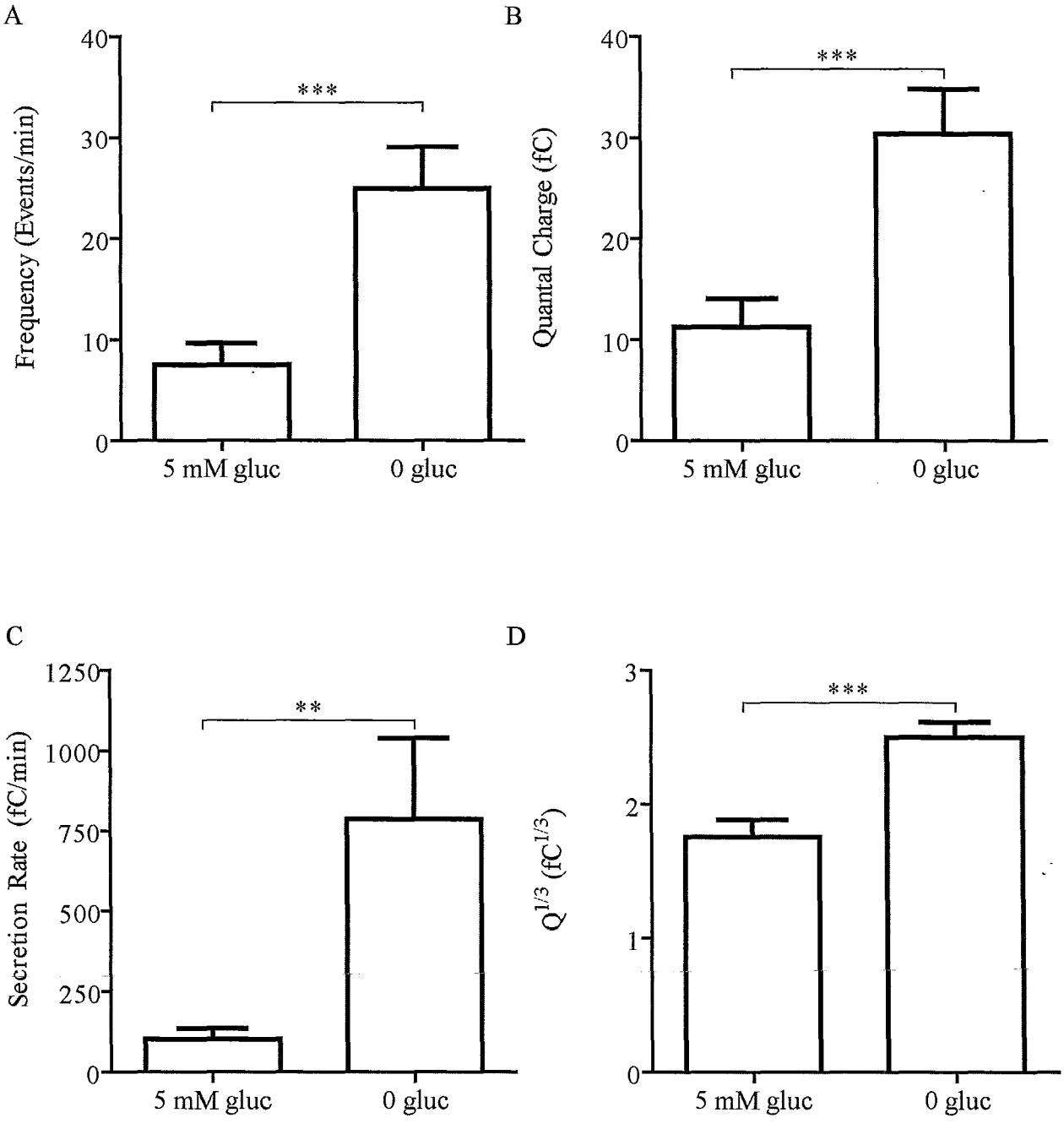

A
Frequency (Events/min)
40
30
20
10
0
***
5 mM gluc
0 gluc
B
Quantal Charge (fC)
40
30
20
10
0
***
5 mM gluc
0 gluc
C
Secretion Rate (fC/min)
1250
1000
750
500
250
0
**
5 mM gluc
0 gluc
D
$Q^{1/3}$ ($fC^{1/3}$)
3
2
1
0
***
5 mM gluc
0 gluc

Figure 2.3. Evidence for a glucose-insensitive population of neonatal AMCs. A, exemplar trace from a cell which failed to respond to aglycemia but displayed robust catecholamine secretion in response to high K^+. B, lack of a change in quantal event frequency in response to aglycemia in these glucose-insensitive cells. Aglycemia evoked no change in mean quantal charge (C) or secretion rate (D) in glucose-insensitive cells. Also, there was no change in $Q^{1/3}$ (a measure of vesicular radius), in these cells (E). Data were compared using a two-way Student's t-test (n = 33 cells).

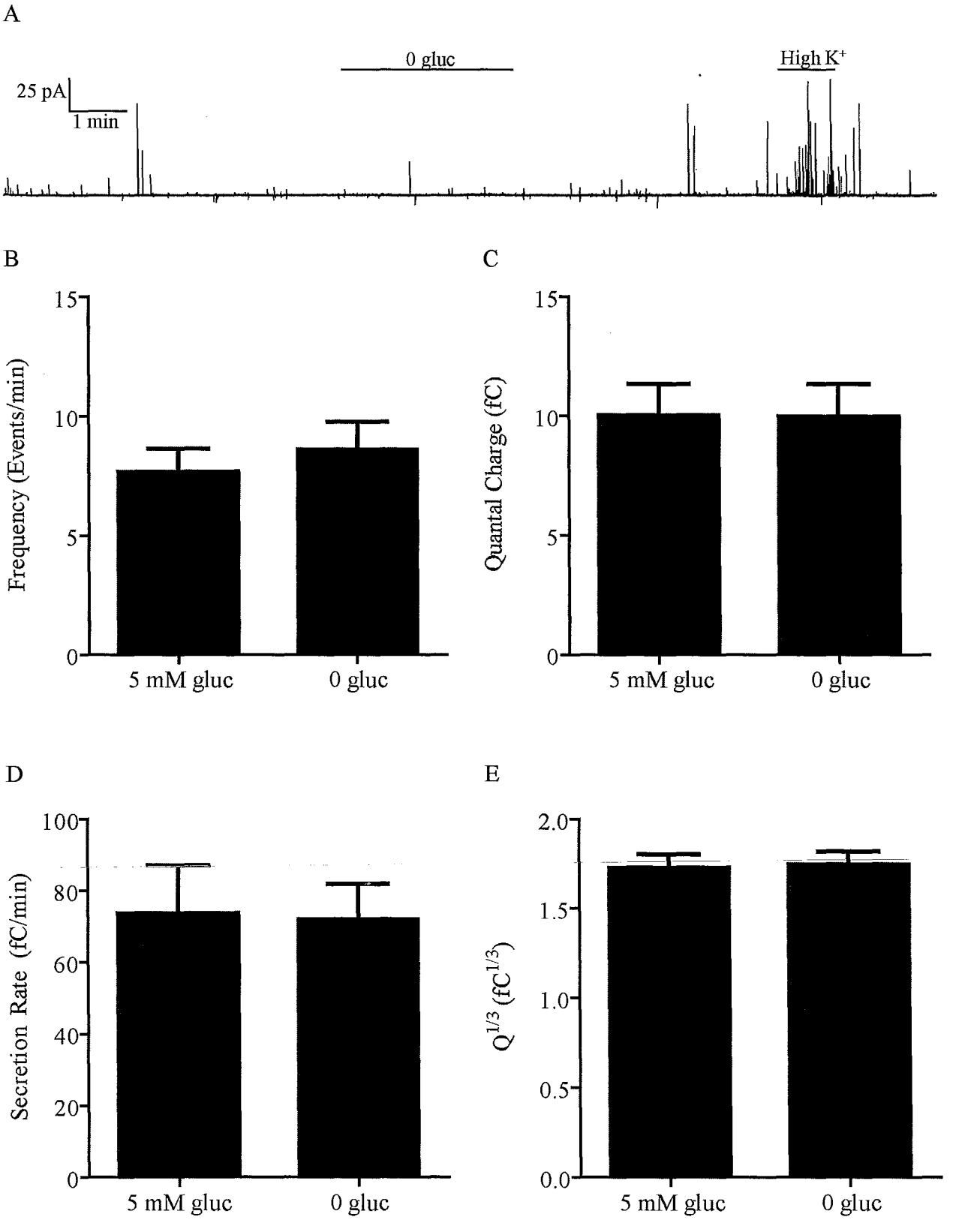
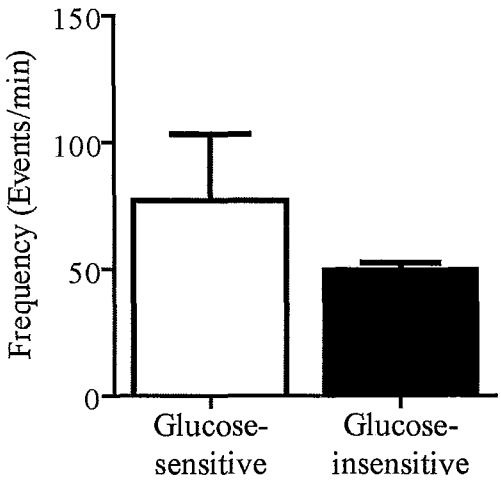

A
0 gluc
High K+
25 pA
1 min
B
Frequency (Events/min)
15
10
5
0
5 mM gluc
0 gluc
C
Quantal Charge (fC)
15
10
5
0
5 mM gluc
0 gluc
D
Secretion Rate (fC/min)
100
80
60
40
20
0
5 mM gluc
0 gluc
E
Q1/3 (fC1/3)
2.0
1.5
1.0
0.5
0.0
5 mM gluc
0 gluc

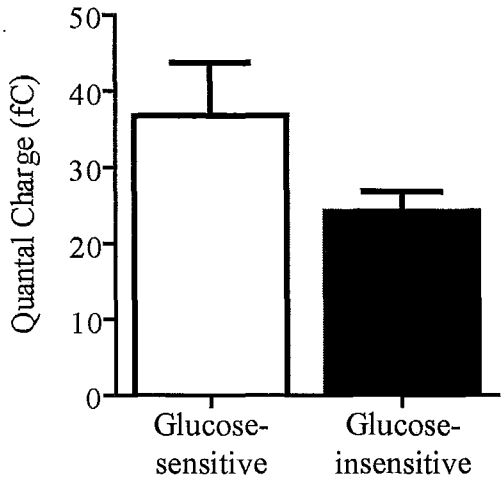
Figure 2.4. Comparison of secretory parameters in glucose-sensitive versus glucose-insensitive AMCs exposed to the depolarizing stimulus, high K^+. A, after exposure to high K^+ there was no significant difference in the frequency of single secretory events in glucose-sensitive (open bars) and glucose-insensitive (closed bars) cells. There was no significant difference in mean quantal charge (B), or secretion rate (C), between glucose-sensitive and glucose-insensitive cells. Also, there was no significant difference in $Q^{1/3}$ between these two populations of cells (D). Data were compared with a two-way Student's t-test (glucose-sensitive cells, n = 17 cells; glucose-insensitive cells, n = 33 cells).

High K^+-evoked Catecholamine Secretion

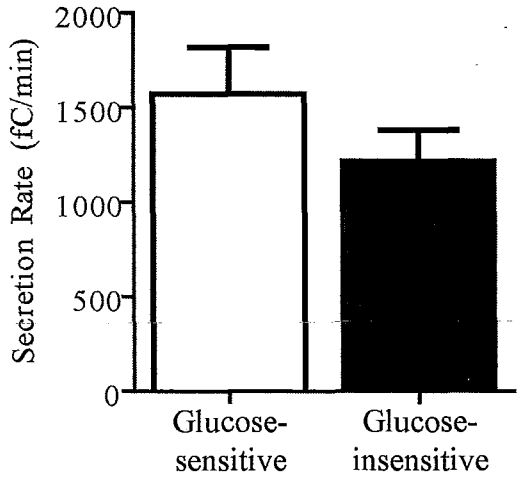
A



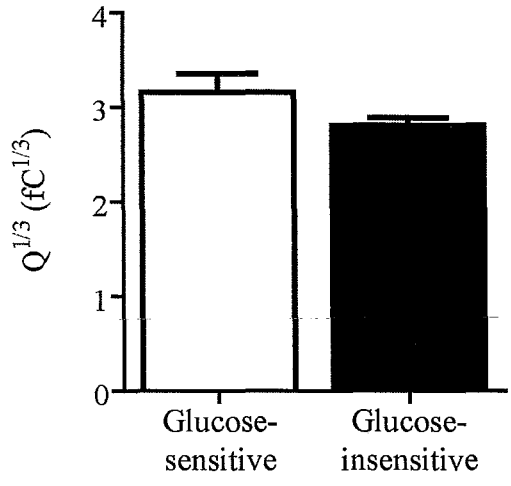
B



C



D



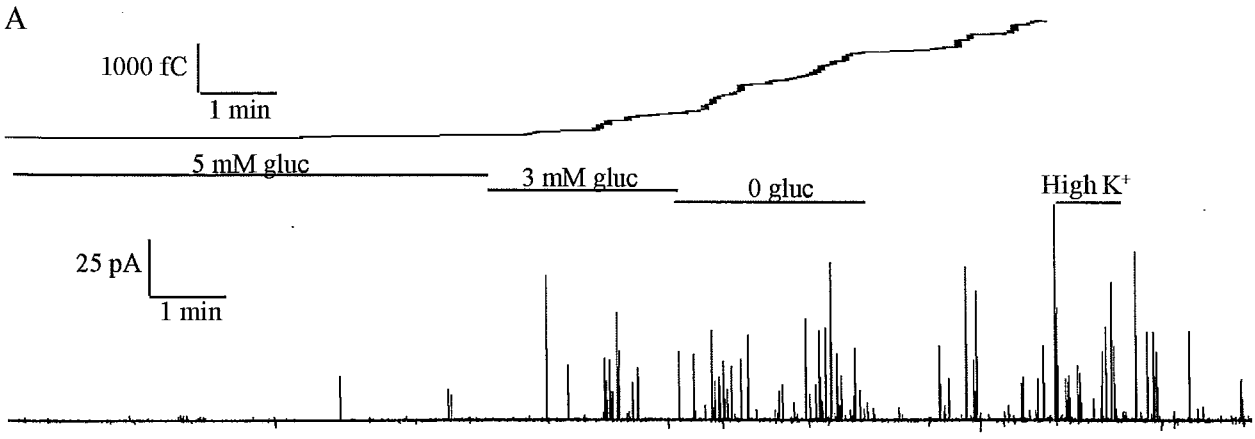
Effects of different concentrations of glucose on catecholamine secretion

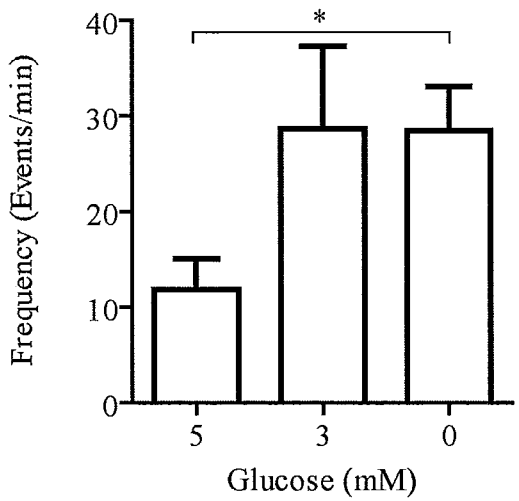
Aglycemia is not a normal physiological stimulus, though it is conceivable that this condition could exist transiently in pathological conditions. It was therefore of interest to test whether neonatal AMCs could mount a detectable catecholamine secretory response to intermediate glucose concentrations (3 mM). In these experiments, the perfusion fluid was switched sequentially from 5 mM glucose (control) to 3 mM glucose (hypoglycemia), and finally to 0 mM glucose (aglycemia), while continuously monitoring catecholamine secretion from the *same* cell (Fig. 2.5A). A secretory response was evident when glucose-sensitive neonatal AMCs were exposed to 3 mM glucose (hypoglycemia); however the response was smaller than that seen during 0 mM glucose (Fig 2.5A, lower). Exposing cells to hypoglycemia led to an apparent increase in the mean frequency of single quantal events, however, this effect was not statistically significant (Fig 2.5B). Though there was no significant difference in mean quantal charge or $Q^{1/3}$ in response to hypoglycemia, there was, as expected, a significant increase in both parameters when the same cells were exposed to aglycemia (Fig 2.5C, E) ($p<0.05$, $n = 7$ cells). Nonetheless, there was a significant increase in total secretion rate when cells were exposed to hypoglycemia (3 mM glucose), compared to control (Fig 2.5D) ($p<0.05$, $n = 7$ cells).

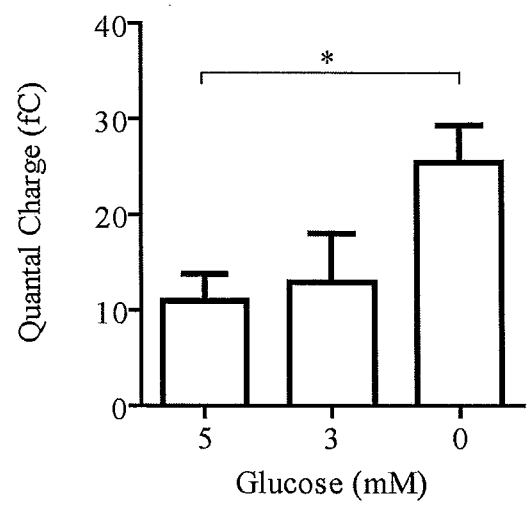
Entry of extracellular Ca^{2+} through VGCC is required for aglycemia-evoked catecholamine release

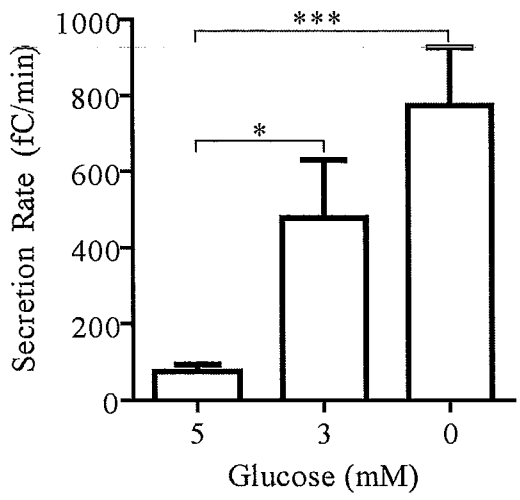
Hypoxia-, acid-, and hypercapnia-evoked catecholamine secretion from neonatal AMC requires the entry of extracellular Ca^{2+} through VGCCs (Mochizuki-Oda *et al.*, 1997; Thompson *et al.*, 1997; Munoz-Cabello *et al.*, 2005; Rico *et al.*, 2005). Likewise, in the present study, the aglycemia-evoked secretory response was reversibly abolished by extracellular application of the

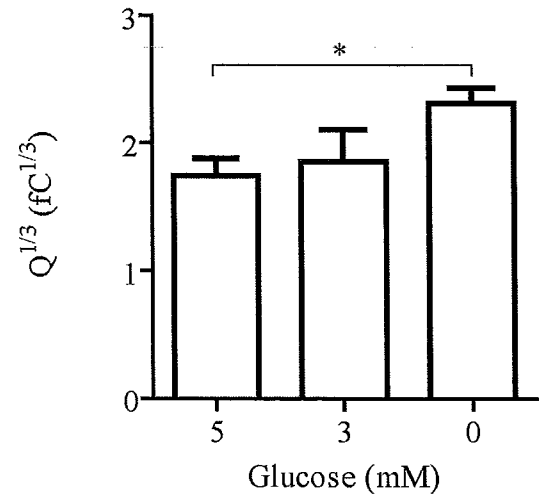
Figure 2.5. Comparison of the effects of different concentrations of glucose on catecholamine secretion from neonatal AMCs. In these experiments the *same* cells were exposed sequentially to 5 mM glucose (control), 3 mM glucose (hypoglycemia), and 0 mM glucose (aglycemia) as illustrated in A; *upper*: cumulative catecholamine secretion corresponding to the cell below; *lower*: exemplar trace of a neonatal AMC showing robust catecholamine secretion in response to both hypoglycemia and aglycemia. There was an apparent, but not statistically significant, increase in mean frequency during hypoglycemia (B) whereas mean quantal charge remained relatively unchanged (C). Nonetheless, relative to control, there was a significant increase in total catecholamine secretion during hypoglycemia and, as expected, aglycemia (D). Unlike aglycemia, however, there was no significant difference in $Q^{1/3}$ in response hypoglycemia relative to control (E). Data were compared with an ANOVA (*$p<0.5$, ***$p<0.001$, $n = 7$ cells).


A
1000 fC
1 min
5 mM gluc
3 mM gluc
0 gluc
High K+
25 pA
1 min


B
Frequency (Events/min)
40
30
20
10
0
*
5
3
0
Glucose (mM)


C
Quantal Charge (fC)
40
30
20
10
0
*
5
3
0
Glucose (mM)


D
Secretion Rate (fC/min)
1000
800
600
400
200
0
***
*
5
3
0
Glucose (mM)


E
Q1/3 (fC1/3)
3
2
1
0
*
5
3
0
Glucose (mM)

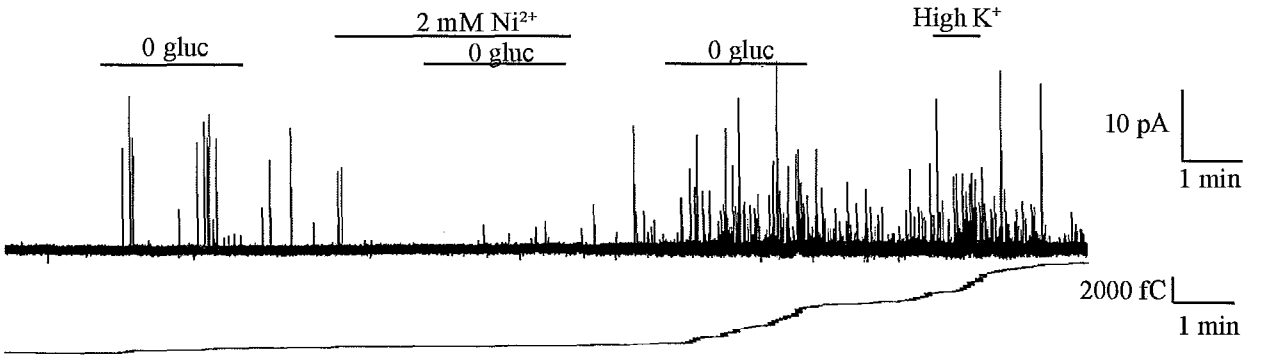
general VGCC blocker Ni^{2+} (2 mM) (Fig 2.6A upper). Ni^{2+} (2 mM) decreased both the aglycemia-evoked increase in event frequency (Fig 2.6B) ($p<0.01$, n = 7 cells) and mean quantal charge to near basal values (Fig 2.6C) ($p<0.05$, n = 7 cells). As a result, Ni^{2+} (2 mM) abolished the aglycemia-evoked increase in catecholamine secretion from neonatal AMC (Fig 2.6D) ($p<0.05$, n = 7 cells). However, there was no statistically significant difference in $Q^{1/3}$ in response to any treatment (Fig 2.6E). In these experiments, the secretory response to aglycemia recovered after wash-out of 2 mM Ni^{2+} (Fig. 2.6A). Indeed, after recovery the response to aglycemia appeared more robust (mean secretion rate = 1993 ± 873.1 fC/min; n = 7 cells) when compared to the original response (mean secretion rate = 773.4 ± 305.9 fC/min; n = 7 cells). This apparent enhancement was not attributable to an effect of Ni^{2+} because it was routinely observed during repeated exposures to aglycemia, even in the absence of Ni^{2+} (data not shown).

To investigate the potential role of L-type VGCC in the secretory response, the L-type channel blocker nifedipine (10 μM) was used. Nifedipine abolished the aglycemia-evoked secretion from neonatal AMC, however recovery after washout of nifedipine was poor (Fig 2.7A upper). Nifedipine completely blocked the increase in event frequency due to aglycemia ($p<0.001$, n = 7 cells) (Fig 2.7B), though there was no statistically significant difference in mean quantal charge (Fig 2.7C). Consequently, nifedipine abolished the agylcemia-induced increase in catecholamine secretion ($p<0.01$, n = 7 cells) (Fig 2.7D). Also, there was no significant change in $Q^{1/3}$ during any of the treatments (Fig 2.7E). These data indicate that the aglycemia-evoked catecholamine secretion requires the influx of extracellular Ca^{2+} through L-type VGCCs.

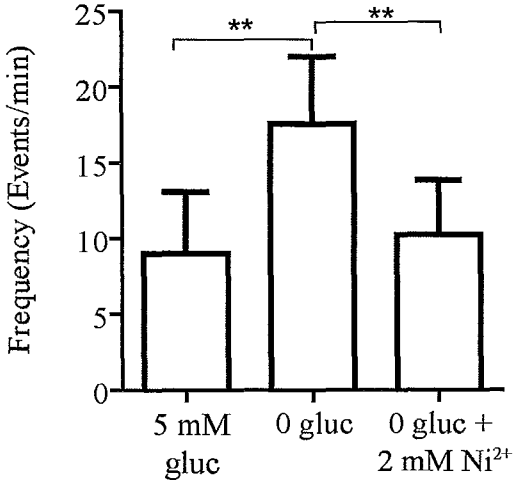
Recently, hypoxia-evoked catecholamine secretion in neonatal AMC has been proposed to require activation of T-type VGCC (Levitsky & Lopez-Barneo, 2009), however this

Figure 2.6. General blockade of VGCCs with 2 mM Ni^{2+} abolishes aglycemia-evoked catecholamine secretion in neonatal AMCs. A, *upper*: representative trace of a neonatal AMC showing aglycemia-evoked catecholamine release before, during, and after 2 mM Ni^{2+}; note reversible blockade of secretory response by 2 mM Ni^{2+}; *lower*: cumulative charge corresponding to the same cell. Ni^{2+} blocked the aglycemia-evoked increase in event frequency (B), mean quantal charge (C), as well as catecholamine secretion rate (D). However, there was no significant difference in $Q^{1/3}$ in response to any treatment (E). Data were compared with an ANOVA (*$p<0.5$, **$p<0.01$, $n = 7$ cells).

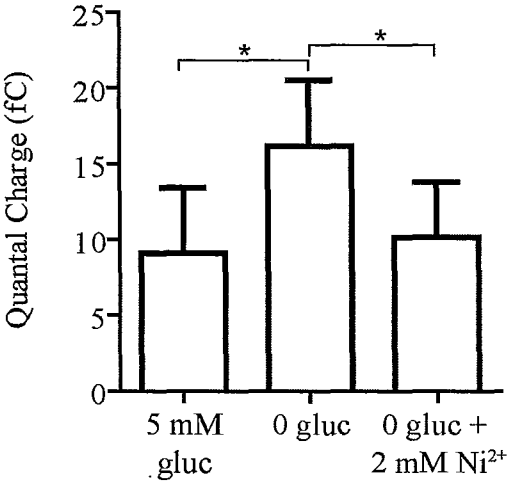
A


0 gluc
2 mM Ni2+
0 gluc
0 gluc
High K+
10 pA
1 min
2000 fC
1 min

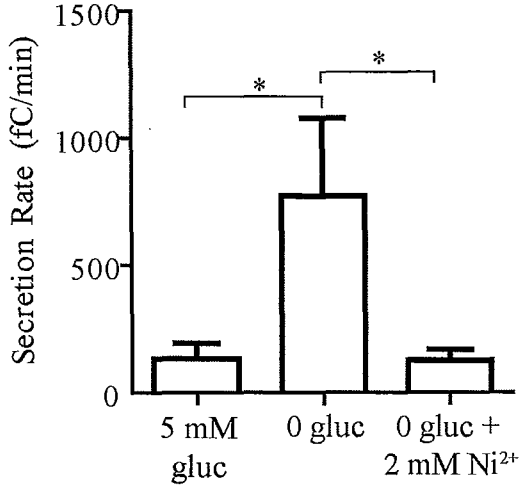
B


Frequency (Events/min)
25
20
15
10
5
0
**
**
5 mM gluc
0 gluc
0 gluc + 2 mM Ni2+

C


Quantal Charge (fC)
25
20
15
10
5
0
*
*
5 mM gluc
0 gluc
0 gluc + 2 mM Ni2+

D


Secretion Rate (fC/min)
1500
1000
500
0
*
*
5 mM gluc
0 gluc
0 gluc + 2 mM Ni2+

E

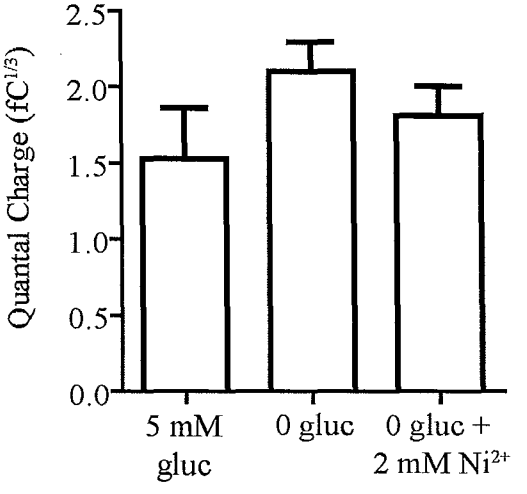
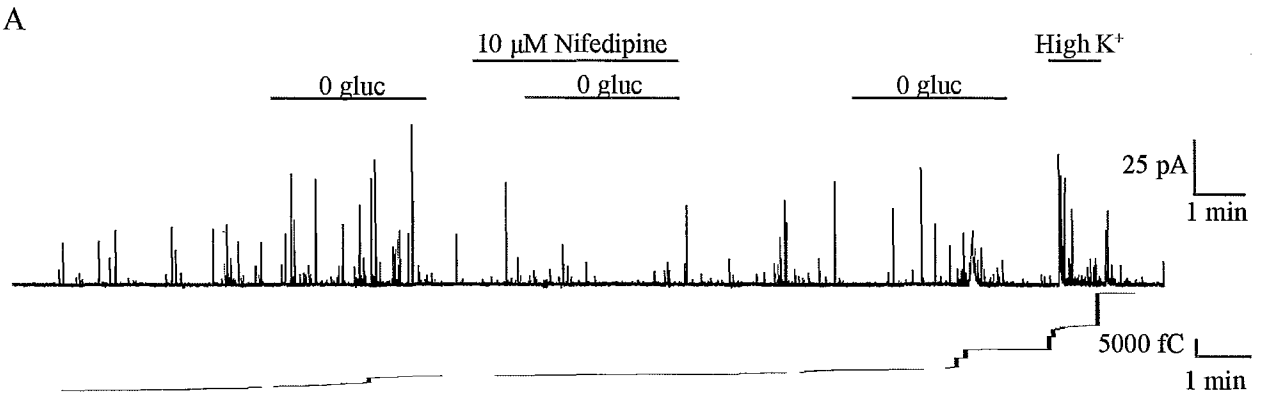
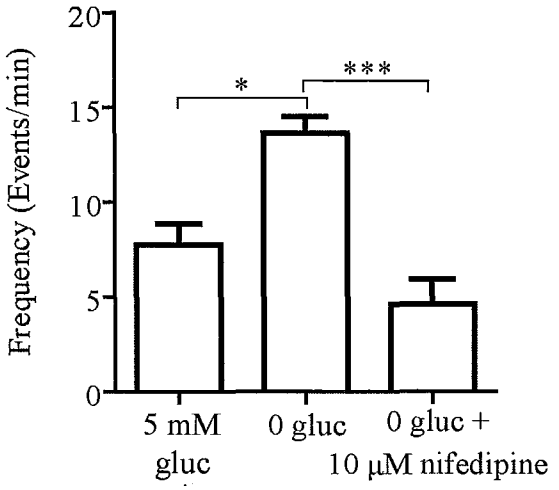
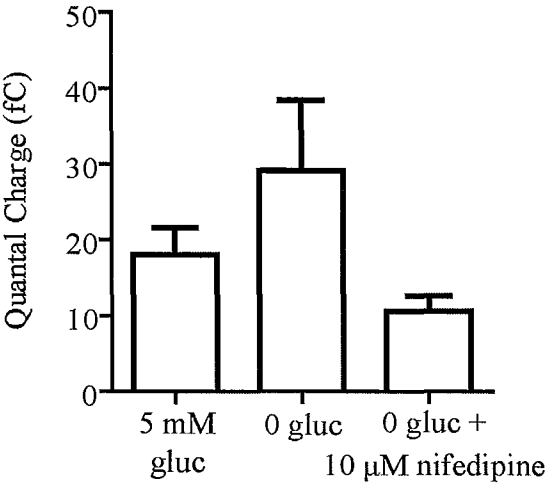
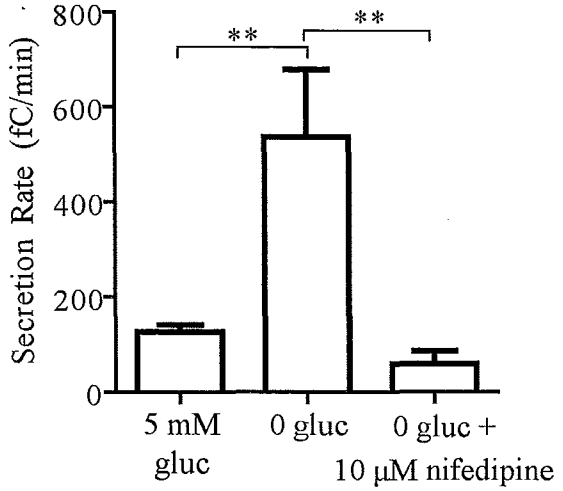

Quantal Charge (fC1/3)
2.5
2.0
1.5
1.0
0.5
0.0
5 mM gluc
0 gluc
0 gluc + 2 mM Ni2+

Figure 2.7. The L-type VGCC blocker nifedipine (10 μM) blocks aglycemia-evoked catecholamine secretion in neonatal AMCs. A, *upper*: example of a glucose-sensitive AMC responding to aglycemia, and this response was blocked by nifedipine. Note repeated application of aglycemia resulted in blunted responses due to poor wash-out of nifedipine. *Lower*: cumulative charge for the cell above. B, nifedipine abolished aglycemia-induced increase in frequency. C, there was no statistically significant change in quantal charge in any group. D, nifedipine abolished the aglycemia-evoked increase in catecholamine secretion. E, there was no significant change in $Q^{1/3}$ in any group. Data were compared with an ANOVA (*$p<0.5$, **$p<0.1$, ***$p<0.001$, $n = 7$ cells).


A
10 μM Nifedipine
High K+
0 gluc
0 gluc
0 gluc
25 pA
1 min
5000 fC
1 min


B
Frequency (Events/min)
20
15
10
5
0
*
***
5 mM gluc
0 gluc
0 gluc + 10 μM nifedipine


C
Quantal Charge (fC)
50
40
30
20
10
0
5 mM gluc
0 gluc
0 gluc + 10 μM nifedipine


D
Secretion Rate (fC/min)
800
600
400
200
0
**
**
5 mM gluc
0 gluc
0 gluc + 10 μM nifedipine

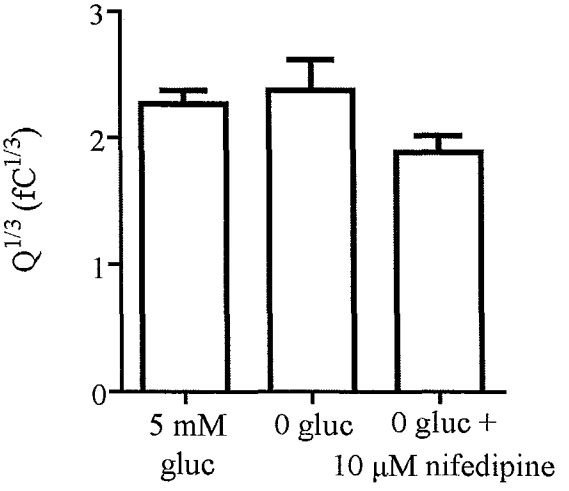
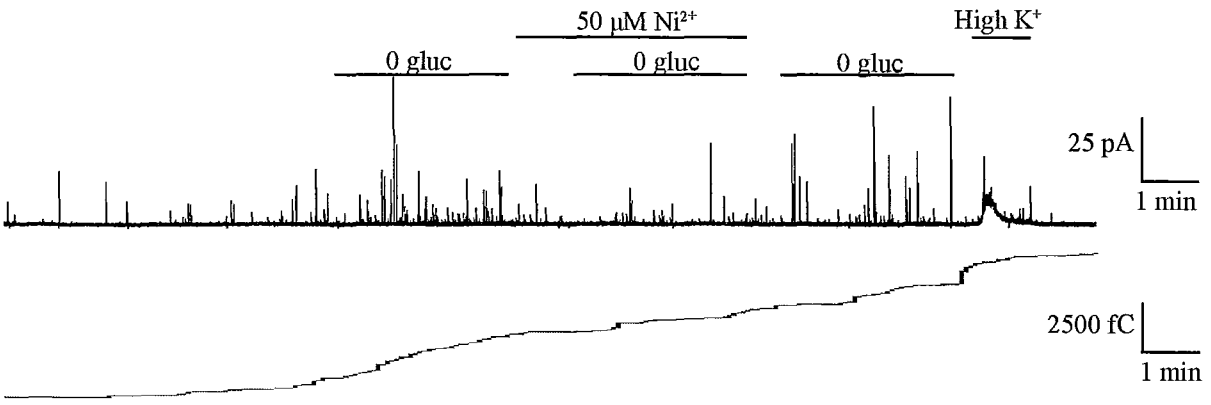
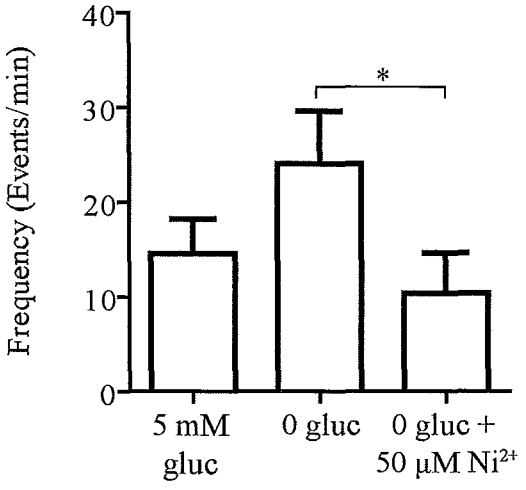

E
Q1/3 (fC1/3)
3
2
1
0
5 mM gluc
0 gluc
0 gluc + 10 μM nifedipine

Figure 2.8. Blockade of T-type VGCCs with 50 μM Ni^{2+} blocks aglycemia-evoked catecholamine secretion. A, *upper*: representative trace of aglycemia-evoked catecholamine secretion in a single neonatal AMC before, during, and after 50 μM Ni^{2+}, which reversibly inhibited the secretory response; *lower*: trace of cumulative secretion corresponding to the cell above. B, although there was no statistically significant change in frequency during aglycemia in this small sample (n= 4), 50 μM Ni^{2+} significantly decreased the frequency of quantal events seen during aglycemia. C, also, 50 μM Ni^{2+} abolished the aglycemia-evoked increase in quantal charge as well as the aglycemia-evoked increase in catecholamine secretion rate (D). However, there was no significant change in $Q^{1/3}$ during any of the treatments (E). Data were compared with an ANOVA (*$p<0.5$, n = 4 cells).

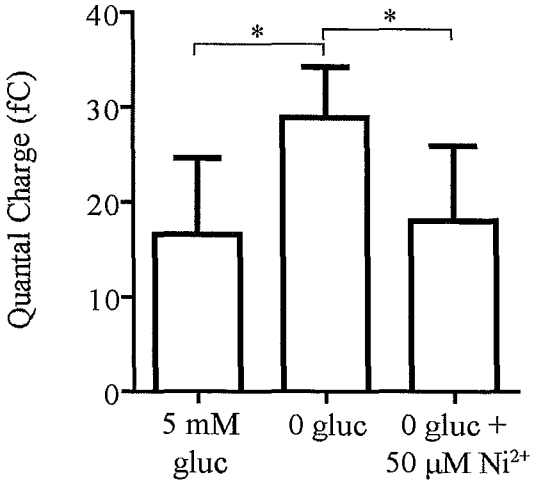
A


50 μM Ni2+
High K+
0 gluc
0 gluc
0 gluc
25 pA
1 min
2500 fC
1 min

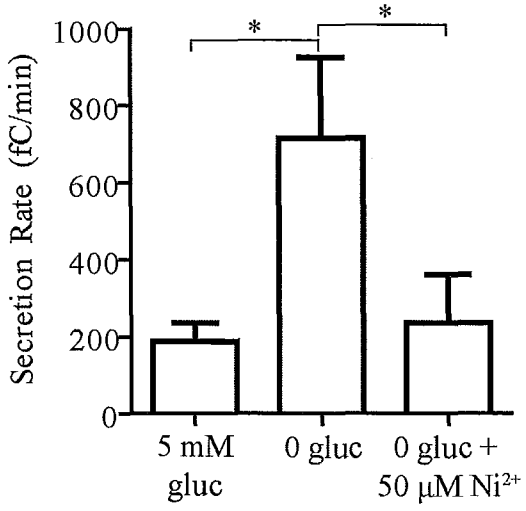
B


Frequency (Events/min)
40
30
20
10
0
*
5 mM gluc
0 gluc
0 gluc + 50 μM Ni2+

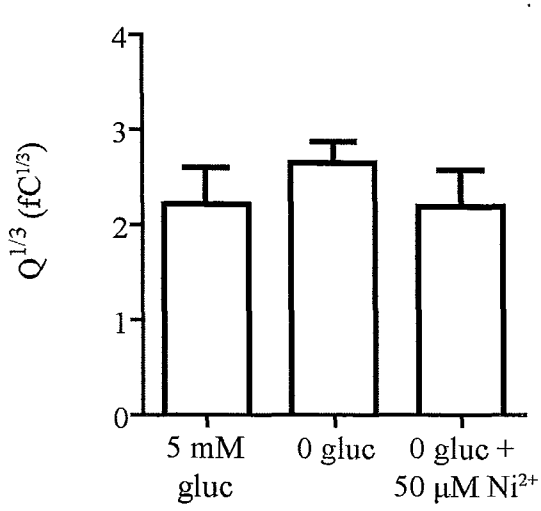
C


Quantal Charge (fC)
40
30
20
10
0
*
*
5 mM gluc
0 gluc
0 gluc + 50 μM Ni2+

D


Secretion Rate (fC/min)
1000
800
600
400
200
0
*
*
5 mM gluc
0 gluc
0 gluc + 50 μM Ni2+

E

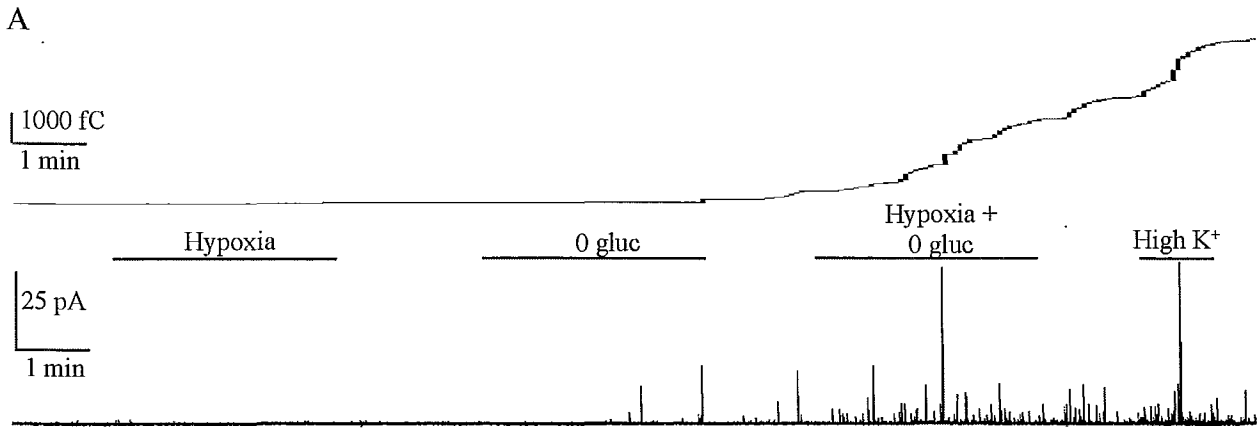

Q1/3 (fC1/3)
4
3
2
1
0
5 mM gluc
0 gluc
0 gluc + 50 μM Ni2+

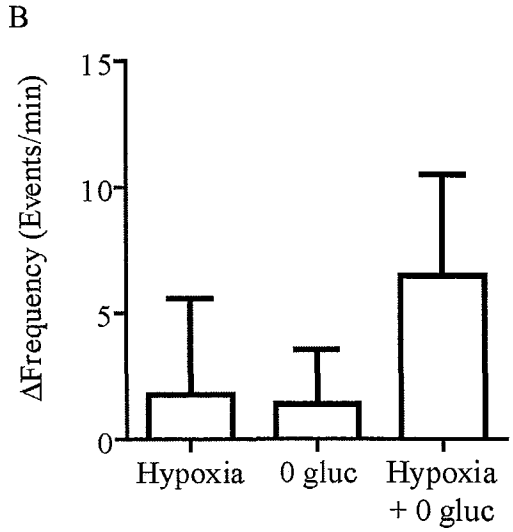
hypothesis remains controversial (Souvannakitti *et al.*, 2010). In the present study, Ni^{2+} (50 μM) at a concentration which blocks T-type, but not L-type, Ca^{2+} currents in neonatal AMCs (Levitsky & Lopez-Barneo, 2009), abolished aglycemia-evoked catecholamine secretion (Fig 2.8A upper). Although 50 μM Ni^{2+} decreased the frequency of quantal events seen during aglycemia ($p<0.05$, n = 4) (Fig 2.8B), the usual increase in frequency that occurs during the switch from normal glucose (5 mM) to aglycemia was not significant in this group probably because of the small sample size (n = 4 cells). Nonetheless, blockade of T-type VGCCs abolished the aglycemia-evoked increase in mean quantal charge ($p<0.05$, n = 4 cells) (Fig 2.8C) as well as the increase in total catecholamine secretion (Fig 2.8D) ($p<0.05$, n = 4 cells). However, there were no statistically significant differences in $Q^{1/3}$ among the various treatments (Fig 2.8E). Thus it appears that both L-type and T-type VGCCs are involved in the secretory response of neonatal AMCs to aglycemia.

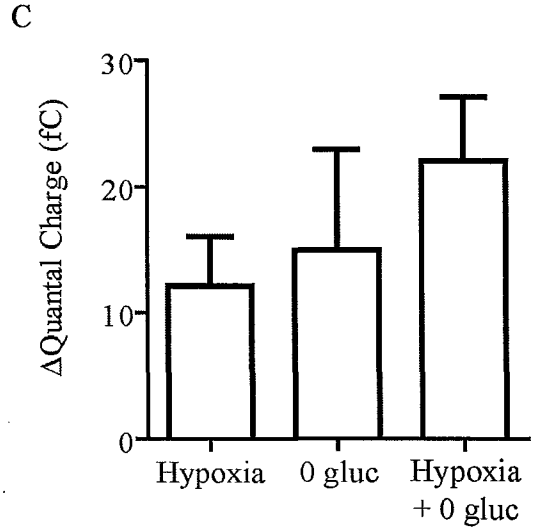
Interactive effects of hypoxia and aglycemia

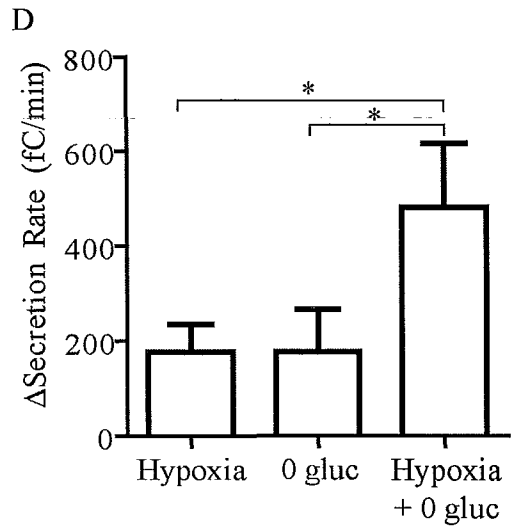
Neonatal AMCs display a robust secretory response when exposed to either low glucose or hypoxia. Therefore, I tested the hypothesis that aglycemia-evoked catecholamine secretion is potentiated when combined simultaneously with hypoxia. In 12 cells studied, 75% (n = 9/12) were hypoxia-sensitive, 58.3% (n = 7/12) were aglycemia-sensitive, and 100% (n = 12/12) were sensitive to the combined stimuli. In some cells there was no detectable secretory response to either hypoxia or aglycemia alone; however, combined application of both stimuli resulted in a robust secretory response that was larger than that due to a single stimulus (Fig 2.9A, lower). Data from these 12 cells were pooled to determine the *change* in secretory parameters (evoked minus basal). Although hypoxia and aglycemia together appeared to increase the frequency of

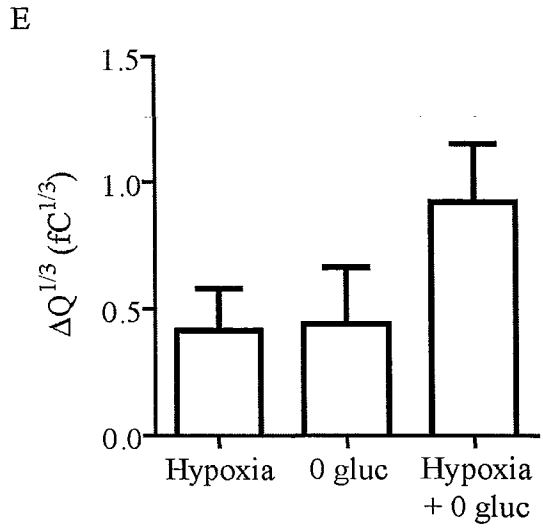
Figure 2.9. The effects of hypoxia and aglycemia, applied separately and together, on catecholamine secretion in neonatal AMCs. A, *upper*: trace of cumulative secretion corresponding to the cell below, *lower*: exemplar trace of a single AMC which failed to respond to hypoxia or aglycemia applied separately, but produced robust secretion in response to the simultaneous application of both stimuli. B, although hypoxia and aglycemia combined appeared to increase event frequency, the effect was not statistically significant. C, there was no statistically significant increase in quantal charge in response to any stimulus. D, hypoxia and aglycemia together significantly increased catecholamine secretion rate in AMCs. E, there was no significant change in $Q^{1/3}$ regardless of the stimulus. Data were compared with an ANOVA (*$p<0.5$, $n = 12$ cells).


A
1000 fC
1 min
Hypoxia
0 gluc
Hypoxia +
0 gluc
High K+
25 pA
1 min


B
ΔFrequency (Events/min)
15
10
5
0
Hypoxia
0 gluc
Hypoxia
+ 0 gluc


C
ΔQuantal Charge (fC)
30
20
10
0
Hypoxia
0 gluc
Hypoxia
+ 0 gluc


D
ΔSecretion Rate (fC/min)
800
600
400
200
0
*
*
Hypoxia
0 gluc
Hypoxia
+ 0 gluc


E
ΔQ1/3 (fC1/3)
1.5
1.0
0.5
0.0
Hypoxia
0 gluc
Hypoxia
+ 0 gluc

quantal events, this effect was not statistically significant (Fig 2.9B). Also, there was an apparent increase in mean quantal charge (Fig 2.9C). Hypoxia alone increased total catecholamine secretion rate by 200.3 ± 65.3 fC/min compared to an increase of 184.4 ± 99.7 fC/min for aglycemia (n = 12 cells) (Fig 2.9D). However, combined application of hypoxia and aglycemia increased catecholamine secretion rate to 561 ± 145.0 fC/min, representing an ~2.8 fold increase over either stimulus alone ($p<0.05$, n = 12 cells). To determine if this interaction was additive or synergistic, I summed the secretion rate for hypoxia alone and aglycemia alone in each cell and then compared that sum to the secretion rate observed in response to combined hypoxia and aglycemia applied to that cell. There was no significant difference between the sum of aglycemia alone and hypoxia alone (360.3 ± 123.8 fC/min) compared to hypoxia and aglycemia combined (357.8 ± 108.5 fC/min), suggesting that these two chemostimuli interacted additively. While there was an apparent increase in $Q^{1/3}$ when cells were exposed to hypoxia and aglycemia together, this effect was not statistically significant (Fig 2.9E).

DISCUSSION

In this study I present evidence for *direct* glucosensing mechanisms in neonatal AMCs, resulting in enhanced catecholamine secretion during exposure to low glucose. Hypoglycemia (3 mM glucose) and aglycemia (0 mM glucose) evoked a robust secretory response in neonatal AMCs and this response required the influx of Ca^{2+} across the plasma membrane facilitated by the opening of L-type and T-type VGCCs. Moreover, I found that many of these glucose-sensitive cells were also hypoxia-sensitive, and that the simultaneous presence of hypoxia potentiated the aglycemia-evoked response.

The robust secretory response from neonatal AMCs was characterized by an increase in the frequency of quantal events and an increase in mean quantal charge. Interestingly,

hypoglycemia seemed to increase mainly quantal frequency, with little change on mean quantal charge. In contrast, hypoxia is known to increase the frequency of single quantal events whereas hypercapnia increases mean quantal size in neonatal AMCs (Munoz-Cabello *et al.*, 2005; García-Fernández *et al.*, 2007a). It is unclear why different stimuli would evoke different patterns of secretion in neonatal AMCs. In neonatal AMCs, hypoglycemia has been demonstrated to cause an increase in action potential frequency (Min Zhang, personal communication), which would be expected to increase the frequency of single quantal events (Zhou & Misler, 1995). Moreover, several intracellular signalling pathways are capable of acutely modifying quantal size; for instance, activation of the cAMP/PKA pathway increases quantal size (Machado *et al.*, 2001) whereas nitric oxide decreases quantal size in AMCs (Machado *et al.*, 2000). In addition, AMCs secrete a variety of paracrine and autocrine mediators including ATP, opioids, and somatostatin which are known to decrease quantal size via activation of the $G_{i/o}$ pathway (Chen *et al.*, 2005). It is plausible that activation of different classes of VGCCs, coupled with their spatial distribution in the plasma membrane may activate different pools of dense-core vesicles, thereby regulating quantal size or frequency. In addition, the distribution of ion channel(s) involved in these responses to asphyxial stimuli may also play a key role in modifying secretion. For instance, hypercapnia activates a non-selective cation channel in neonatal AMCs and influx of Ca^{2+} through this channel may modulate Ca^{2+}_i signalling and modify secretion (Munoz-Cabello *et al.*, 2005).

Aglycemia-evoked secretion requires activation of VGCCs

The influx of extracellular Ca^{2+} through both L-type and T-type VGCCs was required for aglycemia-evoked catecholamine secretion in neonatal AMCs. The requirement for L-type

VGCCs is consistent with the secretory response of these cells to hypoxia and hypercapnia (Thompson *et al.*, 1997; Munoz-Cabello *et al.*, 2005). However, although blockade of T-type VGCC with 50 μM Ni^{2+} abolished hypoxia-evoked secretion in neonatal AMC (Levitsky & Lopez-Barneo, 2009), in a recent study the T-type VGCC blocker mibefradil (10 μM) failed to affect hypoxia-evoked secretion (Souvannakitti *et al.*, 2010). In the latter study, the authors noted that T-type Ca^{2+} channel currents could not be identified in all cells. Therefore the role of T-type VGCC in chemosensing by neonatal AMCs remains controversial. These results could be explained, in part, by the use of different preparations, for example fresh tissue slices (Levitsky & Lopez-Barneo, 2009) versus dissociated cell cultures (Souvannakitti *et al.*, 2010). Though a role for T-type VGCC was demonstrated in AMC cultures in the present study, it is known that the expression of VGCCs in AMCs is exquisitely sensitive to differences in culture technique (Benavides *et al.*, 2004).

Glucose-insensitive cells and interactions between aglycemia and hypoxia

In addition to glucose-sensitive AMCs, a significant proportion of cells failed to respond to aglycemia with increased catecholamine secretion. Despite this observation, the glucose-insensitive population identified in this study mounted a robust high K^+-evoked secretory response, as did their glucose-sensitive counterparts, suggesting that the lack of low glucose sensitivity was not attributable to a defective secretory pathway. It is plausible that these glucose-insensitive cells are still chemosensitive but may require a different stimulus, or a combination of weak stimuli, for detection of evoked catecholamine secretion. Indeed, I found evidence for stimulus interaction in experiments where two stimuli, i.e. hypoxia and aglycemia, were combined. In such cases, hypoxia and aglycemia combined to elicit a more robust

secretory response than either stimulus alone. Moreover a greater proportion of cells responded to the combined stimulus, including cells that failed to respond to separate application of the two stimuli. This may be relevant for catecholamine secretion from AMC *in vivo* because arterial PO_2 (90 mmHg) is much lower than atmospheric (150 mmHg). Moreover, most mammals are exposed to a chronically hypoxic environment *in utero* (PO_2 ~30 mmHg), suggesting that more cells are likely to be sensitive to the combination of these metabolic stimuli *in vivo* (Ream *et al.*, 2008). It should also be noted that perinatal AMCs express ATP-sensitive K^+ channels (K^+_{ATP}) which open during reduced ATP levels (Thompson & Nurse, 1998; Bournaud *et al.*, 2007; Buttigieg *et al.*, 2009), as occurs during both hypoxia and aglycemia, thereby blunting stimulus-induced membrane depolarization and secretion. Indeed, blockade of these K^+_{ATP} channels with glibenclamide in immortalized fetal-derived AMC increased the proportion of cells responding to aglycemia with a detectable increase in Ca^{2+}_i concentration (Piskuric *et al.* 2008). Thus, it is conceivable that relatively high K^+_{ATP} expression levels in some neonatal AMC may have rendered them glucose-insensitive, when using secretion data as a reporter of responsive cells.

Role of Catecholamines in Perinatal Glucose Homeostasis

The counterregulatory hormonal changes that occur in response to hypoglycemia are similar in perinatal mammals and in adults, however the mechanisms underlying these changes differ. In adult mammals, neurons in the lateral hypothalamus sense hypoglycemia and initiate the sympathoadrenal release of catecholamines (Levin *et al.*, 2004). This contrasts with the situation in most mammals (including humans and rats) where splanchnic innervation of the adrenal gland is immature and non-functional at birth (Artal, 1980; Tomlinson & Coupland, 1990). In addition, hypoglycemia increases glucagon release from α cells and decreases insulin

secretion from β cells in the pancreas of perinatal and adult mammals, however neonatal α cells are glucose-insensitive until several weeks after birth (in the rat) (Edwards *et al.*, 1972; Lernmark & Wenngren, 1972; Marliss *et al.*, 1973; Sodoyez-Goffaux *et al.*, 1979). In human newborns, increases in circulating adrenaline and cortisol are the primary hormonal response to hypoglycemia (Jackson *et al.*, 2004). This suggests that in perinatal animals, hypoglycemia acts *directly* on AMCs to initiate catecholamine secretion, as demonstrated in the present study. The released catecholamines could then inhibit insulin release, and initiate glucagon secretion from the pancreas. Consequently, glucagon and catecholamines could both initiate hepatic glycogenolysis to correct perinatal hypoglycemia (Hunter, 1969; Girard *et al.*, 1973b).

Although the focus of my study was glucosensing in neonatal (P0) AMCs, the role of catecholamines in glucose homeostasis *in utero* is well established (Sperling *et al.*, 1984; Girard *et al.*, 1992). Maternal hypoglycemia or occlusion of the umbilical artery, which occurs occasional during development, produces hypoglycemia in the rat fetus (Czikk *et al.*, 2001). Fetal hypoglycemia leads to a surge in circulating catecholamines, and moreover, exogenous catecholamines can increase circulating glucagon and deplete liver glycogen (Girard *et al.*, 1972; Phillippe & Kitzmiller, 1981; Guarner & Alvarez-Buylla, 1992). Furthermore, infusion of insulin into pregnant ewes or their fetuses produces fetal hypoglycemia and a rise in fetal catecholamines (Harwell *et al.*, 1990; Burrage *et al.*, 2009). Also, infusion of catecholamines into the fetus decreases insulin secretion, triggers glucagon release, and leads to a rise in circulating glucose (Jones & Ritchie, 1978; Fowden, 1980; Sperling *et al.*, 1980; Bassett & Hanson, 2000). Critically, fetal adrenergic receptor blockade increases insulin secretion and reduces circulating glucose in the fetus, suggesting that catecholamines play a role in glucose homeostasis in the steady state *in utero* (Leos *et al.*, 2010). In human pregnancies complicated

by maternal diabetes, standard practice involves tight glycemic control to avoid the severe morbidities and mortalities associated with hyperglycemia *in utero* (ter Braak *et al.*, 2002). As a result, bouts of maternal hypoglycemia have increased, which produces fetal hypoglycemia and hypoinsulinemia (Rosenn *et al.*, 1995). This can produce changes in fetal heart rate and movement (Stangenberg *et al.*, 1983; Holden *et al.*, 1984; Langer & Cohen, 1984; Confino *et al.*, 1985; Bjorklund *et al.*, 1996) and increase surfactant in the amniotic fluid (Zapata *et al.*, 1994). These changes suggest that fetal AMCs are capable of responding to hypoglycemia with inappropriately timed catecholamine secretion.

During birth, the transport of wastes and nutrients across the umbilical cord is occluded and blood glucose decreases to a nadir of about 2-3 mM during the first 2 hours of life, before stabilizing at a higher level (Hagnevik *et al.*, 1983; Irestedt *et al.*, 1984; Srinivasan *et al.*, 1986; Tanzer *et al.*, 1997; Marom *et al.*, 2010). This hypoglycemia occurs in concert with hypoxia and acid hypercapnia to trigger the release of catecholamines, which prepares the fetus for extrauterine life. In human neonates, maternal glucose at birth is inversely correlated to the degree of hypoglycemia in the neonate an hour after birth (Srinivasan *et al.*, 1986). In rats, this is due to neonatal hyperinsulinemia and blunted catecholamine and glucagon secretion (Nurjhan *et al.*, 1985). This suggests the presence of hyperglycemia at birth blunts catecholamine secretion and prevents glycogenolysis producing neonatal hypoglycemia. Indeed, in one clinical trial, fetal stimulation of β-adrenoreceptors with terbutaline resulted in significantly higher blood glucose concentrations in newborns delivered by caesarean section (Eisler *et al.*, 1999).

In some newborns, hypoglycemia is more severe or prolonged, thereby increasing the risk of brain damage (Lucas *et al.*, 1988). Risk factors for hypoglycemia include prematurity, newborns of diabetic mothers, and small-for-gestational-age neonates (Williams, 1997). Infants

of diabetic mothers are born with hyperinsulinemia and the hypoglycemia-evoked counterregulatory surge in catecholamines and glucagon is absent (Stern *et al.*, 1968; Bloom & Johnston, 1972). This indicates aberrant catecholamine release from AMCs, and greatly increases the risk of morbidity and mortality in these newborns. Recently, adrenaline has been used to alleviate neonatal hypoglycemia, however, these authors found that intramuscular adrenaline injection was not as safe or as effective as glucose administration, either because of an insufficient dose or differences in the pattern required (Pedersen *et al.*, 2009). Therefore, uncovering the mechanisms of glucosensing in neonatal AMCs may provide a better understanding of the pathogenesis of hypoglycemia and possible therapeutic outcomes.

In conclusion, I have provided evidence for the *direct* intrinsic glucosensing properties of neonatal AMCs. In response to hypoglycemia, many of these cells activate signalling pathway(s) that result in voltage-gated Ca^{2+} entry, and secretion of catecholamines. Moreover, this response is potentiated by the presence of hypoxia. Catecholamines play a pivotal role in perinatal glucose homeostasis, the dysfunction of which increases the risk of perinatal morbidity and mortality. Glucose administration is the only currently accepted method of treating neonatal hypoglycemia. Therefore further work elucidating the mechanisms of glucosensing in neonatal AMCs may provide a better understanding of perinatal glucose homeostasis and lead to other clinical treatments.

CHAPTER 3

Effects of chronic hypoxia on aglycemia-evoked catecholamine secretion from neonatal adrenomedullary chromaffin cells

SUMMARY

During development fetuses are exposed to a chronically hypoxic (Chox) environment, which is essential for proper development. Circulating catecholamines protect the heart during bouts of hypoxia and are required for fetal survival. Here, I investigated the effects of Chox on aglycemia- and high K^+ (30 mM KCl)-evoked catecholamine secretion from neonatal adrenomedullary chromaffin cells (AMCs). After Chox (2% O_2; 24 hrs), cells responded to aglycemia with an augmentation in the frequency of quantal events, quantal charge (Q), $Q^{1/3}$, and secretion rate relative to cells raised under normoxia (Nox; 21% O_2; 24 hrs). Although these parameters, as applied to basal secretion, appeared to be similarly increased after Chox, the effect was not statistically significant. To determine whether the augmentation was specific for aglycemia-evoked secretion, the secretory parameters were compared between Chox and Nox cells exposed acutely to high K^+. Pre-treatment with Chox resulted in no statistically significant change in quantal frequency during high K^+ exposure. However, there was a significant increase in mean quantal charge, $Q^{1/3}$, as well as a substantial increase in secretion rate.

INTRODUCTION

Mammalian embryos develop in a constitutively hypoxic environment. While this is necessary for proper development, severe hypoxia can also lead to a drastic decrease in heart rate (i.e. bradycardia) (Giaccia *et al.*, 2004). Though this bradycardia can in turn lead to prenatal

death, the severity of the effect is blunted by a rise in circulating catecholamines (Ream *et al.*, 2008). Indeed in catecholamine-deficient mice, resulting from knockout of the tyrosine hydroxylase (TH) gene, death occurs due to cardiac failure at embryonic day (E11.5-15), and this phenotype can be rescued by administration of noradrenaline agonists (Zhou *et al.*, 1995). Alternatively, increasing maternal O_2 supply (33-63% O_2) also rescues TH-deficient mice and restores the wild-type phenotype (Ream *et al.*, 2008).

Interestingly, knockout of the transcription factor hypoxia inducible factor (HIF) 2α (HIF-2α) results in the same phenotype as TH null mice (Tian *et al.*, 1998). During chronic hypoxia HIF-2α stabilizes and translocates to the nucleus where it initiates changes in gene expression (Giaccia *et al.*, 2004). During fetal development HIFs promote growth processes such as hematopoiesis, chondrogenesis, and angiogenesis (Tomanek *et al.*, 1999; Schipani *et al.*, 2001; Semenza, 2004), and moreover, HIF-2α is required for the developmental expression of downstream catecholamine biosynthetic enzymes, i.e. DOPA decarboxylase and dopamine-β-hydroxylase, in chromaffin cells (Brown *et al.*, 2009).

The few studies which have examined the effects of Chox on catecholamine secretion have provided conflicting evidence. In the phaeochromocytoma cell line (PC12) Chox augments acute hypoxia-evoked catecholamine secretion via an increase in event frequency and mean quantal size (Taylor *et al.*, 1999). Moreover, Chox upregulated T-type VGCC in *adult* AMCs producing an increase in basal catecholamine secretion (Carabelli *et al.*, 2007). In contrast, hypoxia-evoked catecholamine secretion was attenuated when neonatal rats were reared in a Chox environment (Souvannakitti *et al.*, 2010). Exposure of isolated neonatal AMCs to Chox *in vitro* augmented the acute hypoxia- and high K^+-evoked catecholamine secretion and these effects were partly attributable to the HIF-2α-dependent upregulation of the adenosine 2a

receptor, combined with paracrine effects of released adenosine (Brown, 2009). Chox also upregulated several proteins involved in the packaging of catecholamines into dense-core vesicles in a fetal-derived immortalized chromaffin cell line and as a result the mean quantal charge increased following Chox (Brown & Reyes, unpublished observations) .

Recently, it has been proposed that the canonical transient receptor potential channel (TRPC3) mediates glucosensing in carotid body glomus cells (García-Fernández *et al.*, 2007b). This non-selective cation channel is expressed in neonatal AMCs and in an immortalized chromaffin cell line, and moreover, its expression is upregulated during Chox in a HIF-2α-dependent manner (Stephen Brown, personal communication). Given this evidence, I hypothesised that Chox may augment aglycemia-evoked catecholamine secretion from neonatal AMCs. It was expected that this work would help clarify the role of Chox in catecholamine secretion from neonatal AMCs and aid in understanding the interaction between Chox and aglycemia in the perinatal period.

MATERIALS AND METHODS

Animal Procedures

Animal care and handling was performed as described in Chapter 2.

Cell Culture Method

Preparation of neonatal AMC cell culture was done as described in Chapter 2, with slight modifications. After sister cultures of neonatal AMCs were prepared, half of them were placed in a normoxic (Nox; 21% O_2) incubator with 95% air/5% CO_2, whereas the remainder were

placed a in a hypoxic incubator (2% O_2/5% CO_2/93% N_2). Cells were kept in these incubators for 24 hours before experiments were performed.

Carbon Fiber Amperometry

These experiments were done as described in Chapter 2.

Solutions

Solutions used were described in Chapter 2.

Statistical Analysis

Data represent mean + S.E.M. and were compared with a two-way ANOVA.

RESULTS

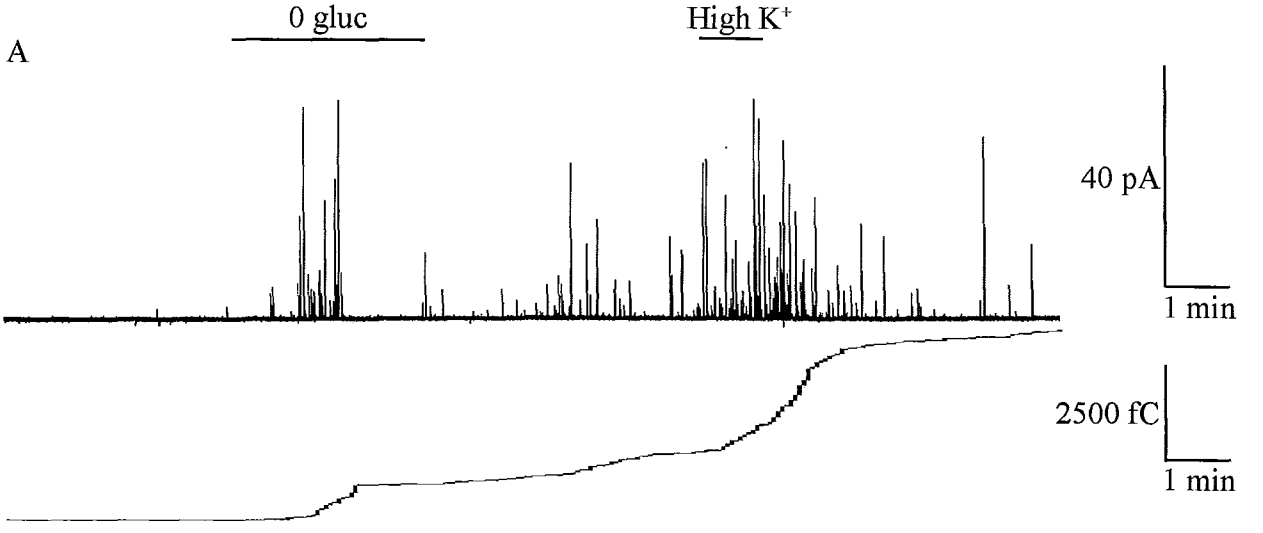
Chronic hypoxia augments aglycemia-evoked secretion

As discussed in Chapter 2, and further illustrated in Figure 3.1A (upper) aglycemia led to a robust secretory response from neonatal AMCs cultured in normoxia (Nox; 21% O_2; 24 hrs). This resulted in a substantial increase in total catecholamine secretion per unit time as measured by amperometry (Fig 3.1A lower). As exemplified in Figure 3.1B (upper), neonatal AMCs cultured for 24 hr under chronic hypoxia (Chox; 2% O_2; 24 hrs) showed a marked potentiation in the secretory response to aglycemia, resulting in a catecholamine secretory rate that was even larger than that seen for Nox cells (Fig 3.1B lower; note different fC scales in A and B).

Relative to basal, aglycemia-evoked a ~2.4 fold increase in quantal frequency in Nox and Chox cells (Fig 3.2A, $p<0.001$). However, the magnitude of basal (5 mM) and aglycemia-

Figure 3.1. Aglycemia- and high K^+ -evoked secretory responses in AMCs cultured in Nox and Chox. A, *upper*: a representative trace of a single normoxic (Nox; 21% O_2, 24 hrs) neonatal AMC showing a robust secretory response when exposed acutely to aglycemia and high K^+ (30 mM); *lower*: trace shows cumulative charge, indicative of total catecholamine secretion, corresponding to the cell above. B, *upper*: example of a chronically hypoxic (Chox; 2% O_2; 24 hrs) neonatal AMC showing robust catecholamine secretion to aglycemia and high K^+, *lower*: cumulative charge corresponding to the cell above.

Nox


A
0 gluc
High K+
40 pA
1 min
2500 fC
1 min

Chox

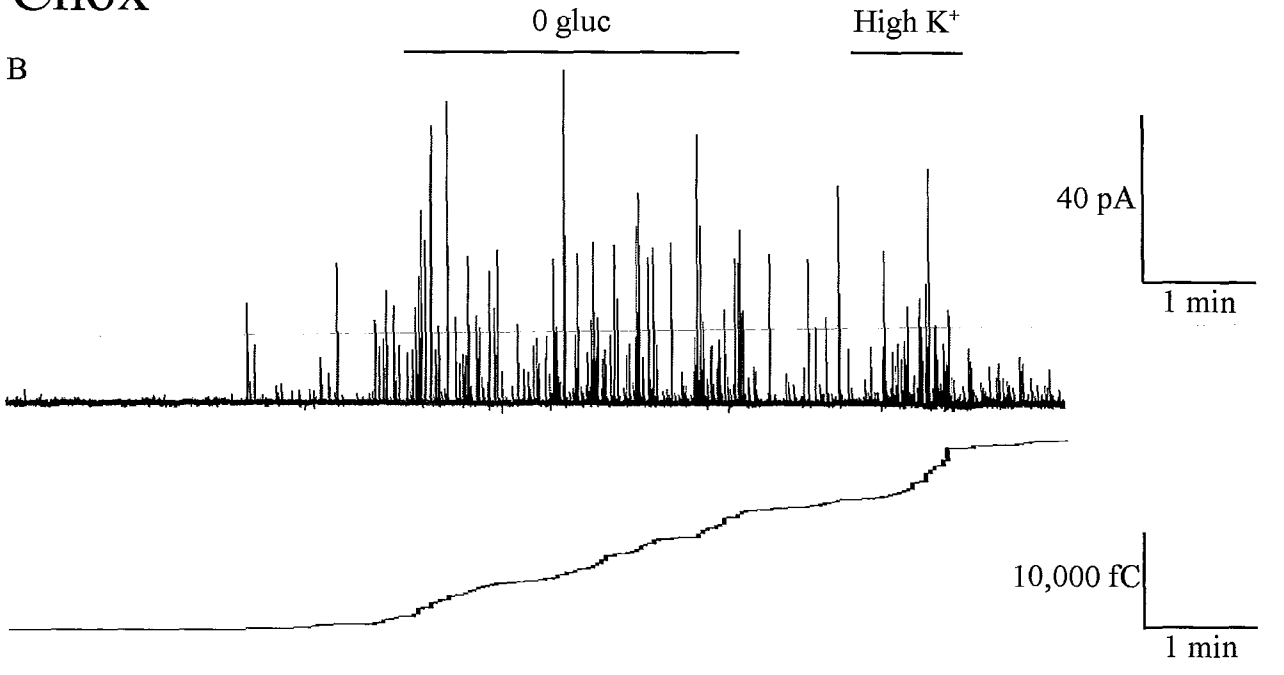
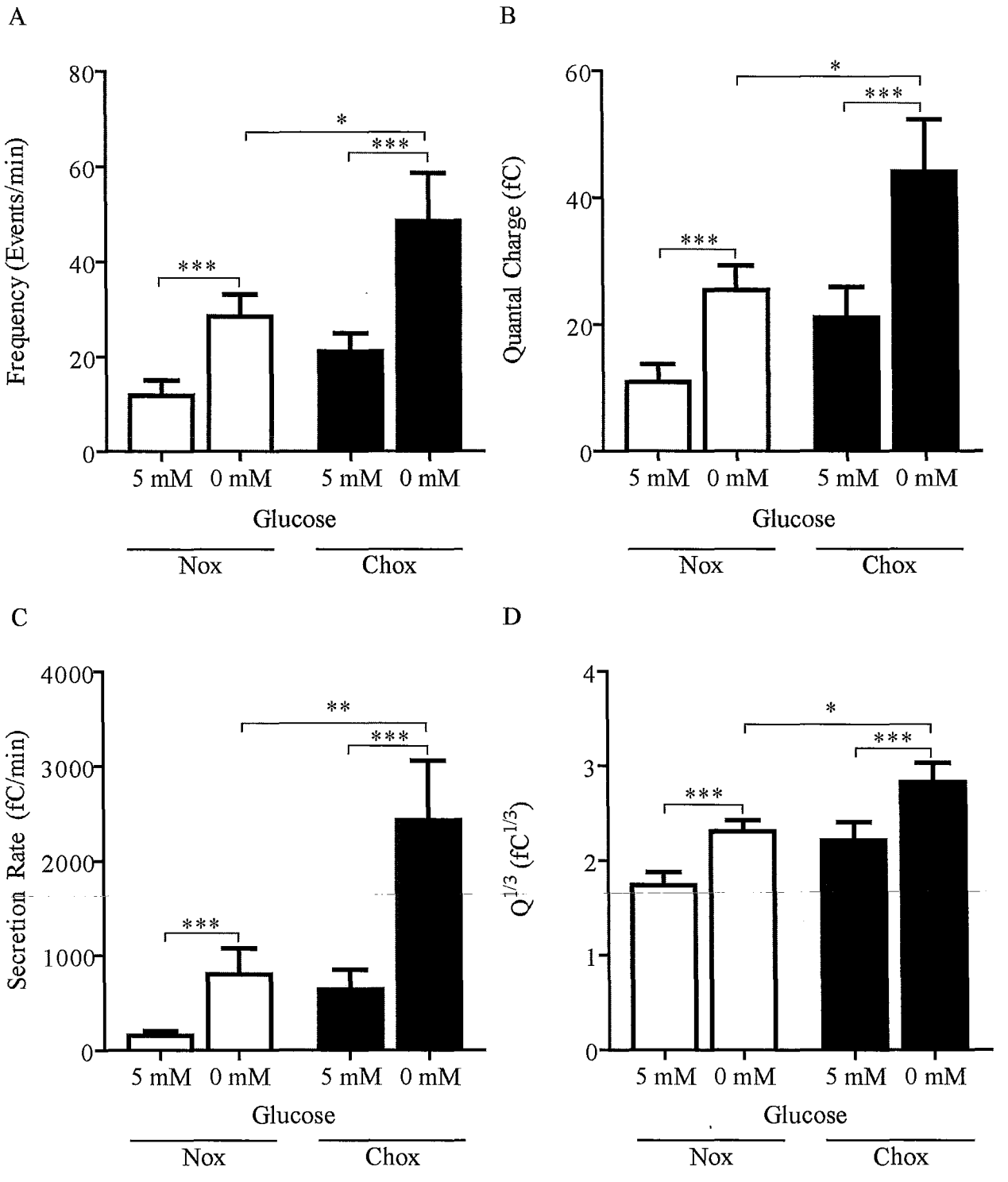

B
0 gluc
High K+
40 pA
1 min
10,000 fC
1 min

Figure 3.2. Comparison of aglycemia-evoked secretory parameters in neonatal AMCs grown in normoxia (Nox) versus chronic hypoxia (Chox). A, quantal event frequency in Nox versus Chox cells. B, mean quantal charge measured in Nox versus Chox cells. Comparison of catecholamine secretion rate (C) and $Q^{1/3}$ (D) in cells cultured in Nox versus Chox. All data were obtained from sister cultures of AMCs grown in Nox (21% O_2, 24 hrs; n = 15) or Chox (2% O_2, 24 hrs; n = 9). Data were analyzed with a two-way ANOVA (in group comparison, i.e. 5 mM glucose in Nox vs. 0 mM glucose in Nox, and between group comparison, i.e. 5 mM glucose in Nox vs. 5 mM glucose in Chox) (*$p<0.05$, **$p<0.01$, ***$p<0.001$).


A
Frequency (Events/min)
80
60
40
20
0
*
***
***
5 mM 0 mM 5 mM 0 mM
Glucose
Nox
Chox
B
Quantal Charge (fC)
60
40
20
0
*
***
***
5 mM 0 mM 5 mM 0 mM
Glucose
Nox
Chox
C
Secretion Rate (fC/min)
4000
3000
2000
1000
0
**
***
***
5 mM 0 mM 5 mM 0 mM
Glucose
Nox
Chox
D
$Q^{1/3}$ ($fC^{1/3}$)
4
3
2
1
0
*
***
***
5 mM 0 mM 5 mM 0 mM
Glucose
Nox
Chox

evoked frequency was ~1.7 fold higher in Chox cells compared to Nox-treated cells. Overall, when exposed to aglycemia Chox-treated cells had a statistically significant increase in frequency compared to Nox cells exposed to aglycemia ($p<0.05$).

Likewise, quantal charge increased ~2.2 fold in response to aglycemia in Nox cells and Chox-treated cells ($p<0.001$) (Fig 3.2B). Furthermore, the mean quantal charge appeared higher in Chox cells exposed to 5 mM glucose compared to Nox cells exposed to 5 mM glucose; however this was not statistically significant. Moreover, when both groups of cells were exposed to aglycemia the mean quantal charge was ~1.7 fold higher in Chox cells than in Nox cells ($p<0.05$). Similarly, $Q^{1/3}$ increased when both groups of cells were exposed to aglycemia ($p<0.001$), and Chox augmented $Q^{1/3}$ only when cells were exposed to aglycemia ($p<0.05$).

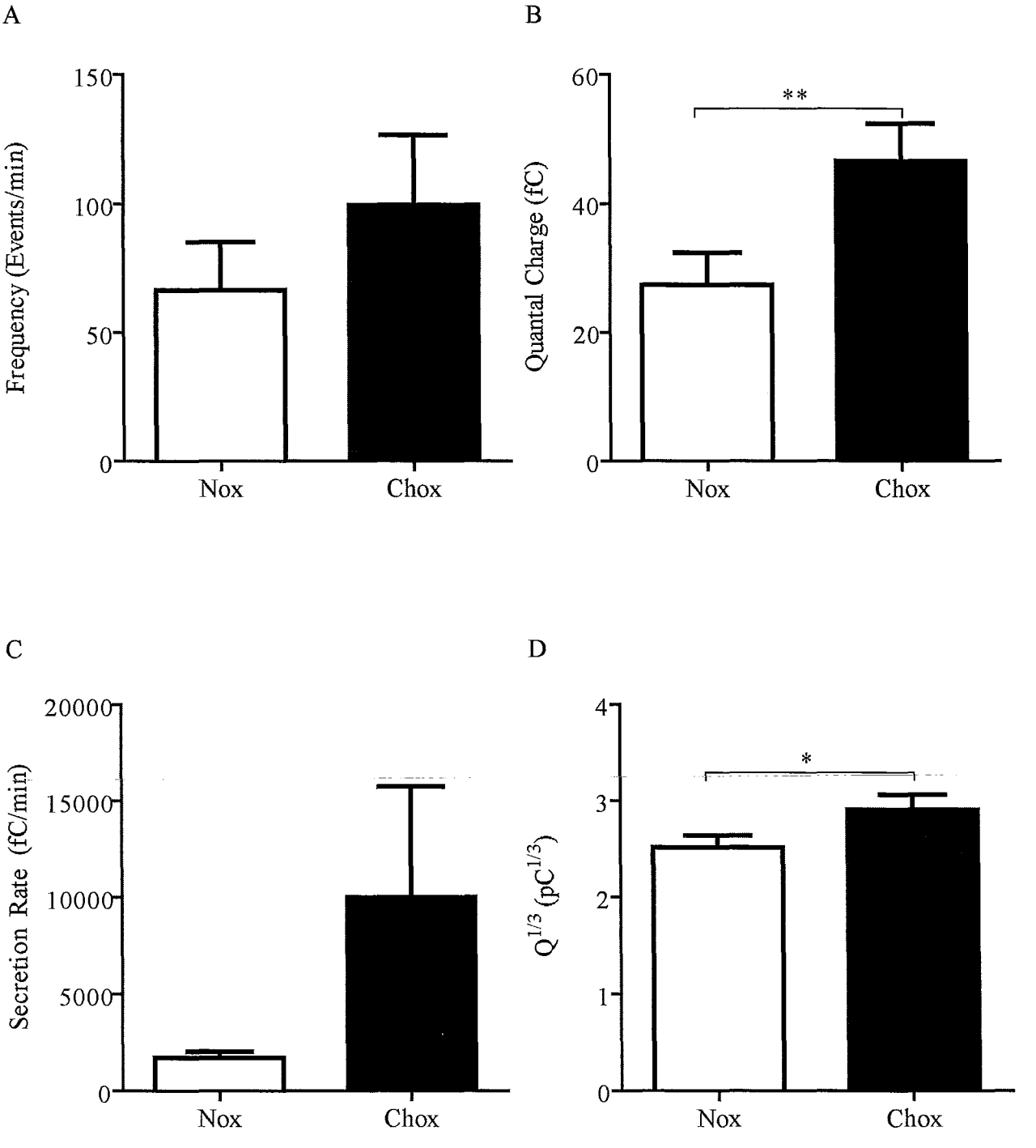
Changes in secretion rate (fC/min) reflect the combination of changes in both frequency and quantal charge (Fig 3.2C). Aglycemia increased the secretion rate ~5.3 fold in Nox cells and ~3.8 fold in Chox cells ($p<0.001$, two-way ANOVA). Though basal secretion was ~4 fold higher in Chox cells compared to Nox cells, this effect was not statistically significant. However, aglycemia-evoked secretion was significantly higher in Chox-treated cells compared to Nox-treated cells ($p<0.01$, two-way ANOVA).

Chronic hypoxia modifies high K^+-evoked secretion from neonatal AMC

Chox may increase secretion generally in neonatal AMC or in response to a specific stimulus. In the first case, secretion could increase in response to any stimulus because of upregulation of the adenosine 2a receptor combined with enhanced autocrine effects of adenosine, and/or upregulation of proteins which regulate quantal size (Brown, 2009). On the other hand, Chox

Figure 3.3. Comparison of high K^+-evoked secretory parameters in neonatal AMCs cultured in Nox versus Chox. A, there was no significant difference in quantal event frequency between Chox and Nox cells, exposed to the depolarizing stimulus high K^+ (30 mM). By contrast, there was a significant increase in mean quantal charge (B), and $Q^{1/3}$ (D), in AMCs cultured in Chox relative to Nox controls ($p<0.01$). C, there appeared to be a large (although statistically non-significant) increase in secretion rate in neonatal AMCs cultured in Chox compared to Nox controls. Data were obtained from sister cultures of AMCs cultured in Nox (21% O_2, 24 hrs; n = 15) versus Chox (2% O_2, 24 hrs; n = 9), and compared using two-way Student's t-test (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

High K^+-evoked Catecholamine Release



may change the expression of proteins which are intimately involved in the aglycemia-evoked response. To test whether the observed increase in secretion after Chox is general or stimulus-specific, I compared high K^+-evoked secretory parameters between Nox and Chox cells.

There was no significant difference in the frequency of high K^+-induced quantal events recorded from Nox and Chox cells (Fig 3.3A), however there was a significant increase in mean quantal charge after Chox (Fig 3.3B; $p<0.01$). Moreover, $Q^{1/3}$ was significantly higher in Chox compared to Nox cells during high K^+ exposure (Fig. 3.3D; $p<0.05$). These data are consistent with evidence that proteins involved in packaging catecholamines into vesicles are upregulated in chromaffin cells during Chox (Brown, 2009). Although the mean high K^+-evoked secretion rate (fC/min) was ~6 fold higher in Chox compared to Nox cells, the effect was not statistically significant, presumably reflecting the large variation in the magnitude of high K^+-evoked secretion among different cells.

DISCUSSION

In this study Chox caused a marked potentiation of aglycemia-evoked catecholamine secretion from neonatal AMCs, mediated by an increase in both frequency and size of quantal events. Several mechanisms may mediate the increase in quantal size, such as greater packaging of catecholamines in vesicles or more complete vesicular emptying during release. Indeed, Chox was found to upregulate a number of genes involved in catecholamine metabolism, packaging and secretion, in a fetal-derived immortalized AMC line including the vesicular monoamine transporters (VMAT1 &2) which help concentrate catecholamines in dense cored vesicles (Brown, 2009). Moreover, after Chox there was an augmentation of the hypoxia-evoked elevation of Ca^{2+}_i in these immortalized AMCs (Brown and Reyes, personal communication),

which may activate different pools of vesicles or lead to more complete release. This increase in Ca^{2+} influx has been proposed to be mediated in part by increased expression of the adenosine 2a receptor combined with the paracrine effects of released adenosine. Activation of this paracrine pathway was recently shown to result in an increase in mean quantal charge in neonatal AMCs, though frequency remained unchanged (Brown & Reyes, unpublished observations). There also remains the possibility that upregulation of T-type VGCC following Chox could also increase secretion, as demonstrated for *adult* chromaffin cells (Carabelli *et al.*, 2007), however this remains to be tested in neonatal AMC.

Although the signal transduction pathway that mediates glucosensing in neonatal AMC is incompletely understood, it is conceivable that TRPC3 channels are involved. These non-selective cation channels have been proposed to underlie glucosensing in the related carotid body glomus cell (García-Fernández *et al.*, 2007b), and they are expressed in perinatal AMCs (Stephen Brown, personal communication). Moreover, TRPC3 channel expression is upregulated ~3 fold at the mRNA level during Chox (Stephen Brown, personal communication) and opening of these channels during aglycemia could promote a secondary source of Ca^{2+} entry. This could partly explain why aglycemia increases both the frequency and size of quantal events whereas the depolarizing stimulus high K^+ only increases quantal size.

A great deal more work is required for a clearer interpretation of the data presented here. The proteins involved in glucosensing in neonatal AMCs need to be elucidated before changes in their expression pattern can be linked to secretory changes following exposure to chronic hypoxia.

APPENDIX I

Problems encountered during this study

Beginning in Nov/Dec 2009 the glucosensitivity of AMCs was lost and all neonatal AMCs responded like glucose-insensitive cells (Chapter 2). This loss of chemosensitivity was confirmed using Fura-2 Ca^{2+} imaging (Nikol Piskuric, personal communication), patch clamp (Min Zhang, personal communication), and carbon fibre amperometry. Furthermore, the responses to other chemostimuli like hypoxia were attenuated (Min Zhang, personal communication). Moreover, some juvenile AMCs which are considered weakly chemosensitive responded robustly to aglycemia (Fig I.1A upper). This response was characterized by an increase in the frequency (Fig I.1B), quantal charge (Fig I.1C), secretion rate (Fig I.1D), and $Q^{1/3}$ (Fig I.1E). To test if this response might be the same as the aglycemia-evoked responses in neonates I tested the Ca^{2+}-dependence of secretion. This response was not blocked when Ca^{2+} was removed from the saline solution (Fig I.2A upper). Indeed, the frequency and secretion rate was greater when Ca^{2+} was removed (Fig I.2B, D), indicating a store-operated mechanism. Moreover, in this laboratory the oxygen-sensing carotid body glomus cells and aortic body glomus cells are responding to reoxygenation (Nikol Piskuric, personal communication). These results are inconsistent with whole-animal studies and single-cell studies performed in this laboratory. As such, I considered them artifacts (perhaps resulting from unknown changes in the animals' hormonal balance, their rearing environment, or laboratory environment) and therefore, attempted to change components of the AMC preparation in order to restore chemosensing.

Since the temperature control of the Animal Care Facility fluctuates widely, we tried keeping our rats in the Animal Care Facility in the Health Sciences Centre. In addition, we

ordered pregnant rats from Harlan and kept them in the Health Sciences Center's Animal Care Facility. New glassware and reagents were purchased for cell culture preparation and saline preparation. Cells were cultured in MAH medium and modified DMEM medium (See Appendix IV). Moreover, our flow hoods were professionally decontaminated. Numerous problems with the incubators had occurred; the CO_2 sensor broke and had to be replaced. Then, the fan broke caused CO_2 levels in the incubator to vary widely; this was subsequently fixed. A new incubator was purchased and its fan broke and had to be fixed.

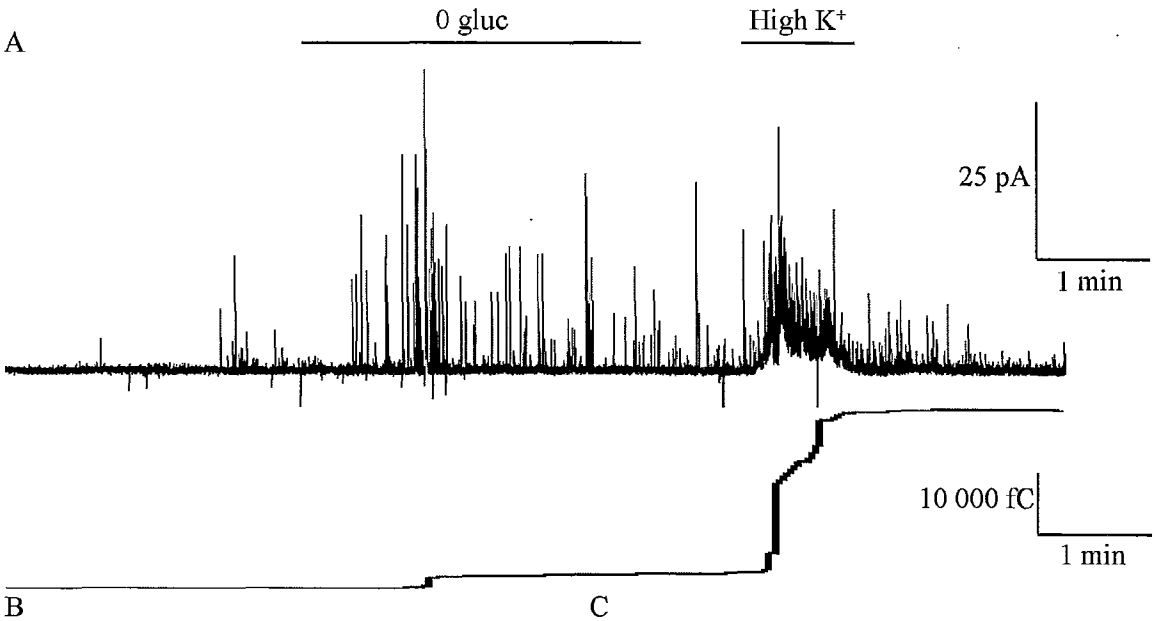
Before these problems arose, we had recently received an immortalized cell line of pancreatic β-cells from a collaborator, which we thought might have contaminated our cultures with mycoplasma. DAPI-staining and mycoplasma detection kit (MycoAlert Kit, Lonza) were negative for mycoplasma. RT-PCR was negative when performed according to the method of Hopert *et al.*, (1993); however it was positive when performed according to the method suggested by Dr. David W. Andrews (Shaima Salman, personal communication).

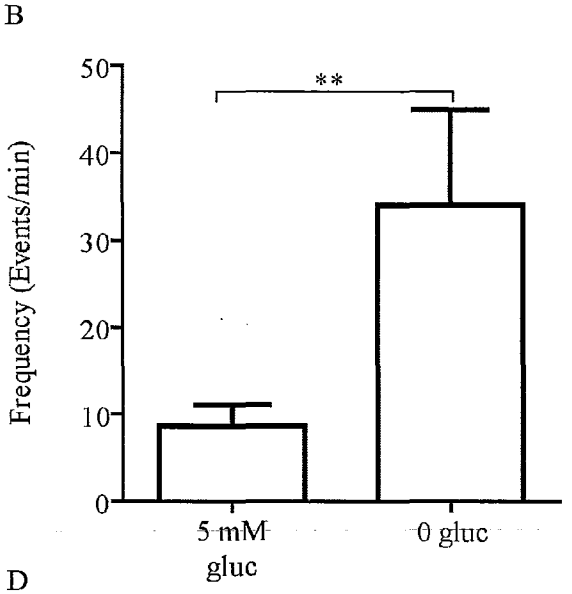
The H_2O in the Life Sciences Building contains dangerously high concentrations of lead (Pb^{2+}), and I could not be certain if the reverse-osmosis machine on the 4^{th} floor worked or not. Therefore, the reverse-osmosis machine on the 5^{th} floor was used for most experiments. In addition, H_2O was obtained from several other sources (Dr. Chris Wood's Lab and the Health Science Centre). Moreover, I purchased cell culture grade water to make saline.

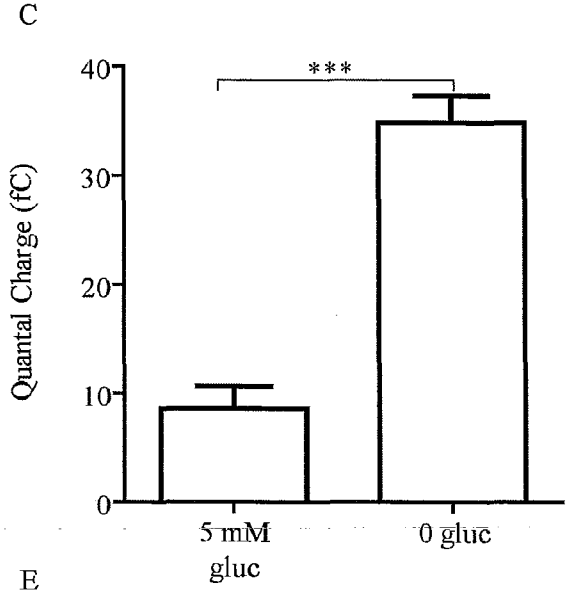
Because changing the above mentioned materials had no effect on glucosensitivity a 3 day lab cleanup was performed. Anything which could not be autoclaved or soaked in EtOH or Roccal was thrown out. For the two week period following the cleanup, glucosensitivity was restored; however, these responses were only transient and could not be measured after 3 weeks.

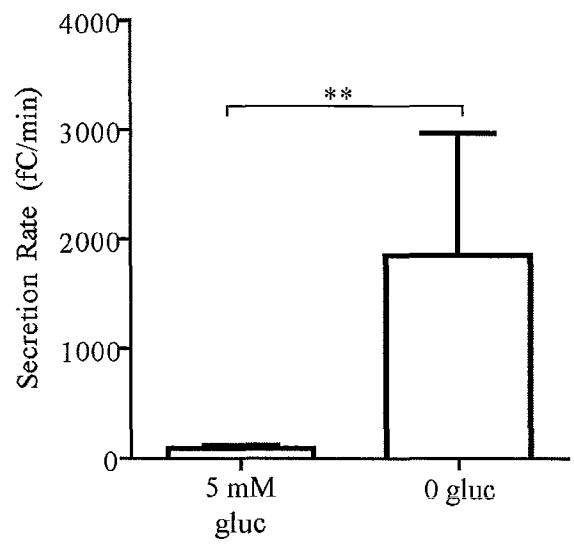
During the Christmas break the lab was shut down and a brief cleanup was performed before the lab was left empty for 7-10 days. Responses were not observed after this time.

Figure I.1. Aglycemia-evoked secretory responses from juvenile AMCs. A, *upper*: representative trace of a juvenile AMC with a robust secretory response to aglycemia, *lower*: cumulative charge corresponding to the cell above. B, juvenile AMCs underwent an increase in event frequency when exposed to aglycemia. This corresponded with an increase in quantal charge (C) and catecholamine secretion rate (D) during aglycemia. E, there was no statistically significant difference in $Q^{1/3}$ in response to aglycemia. Data represent mean + S.E.M. n = 8 cells, data compared using Student's t-test.


A
0 gluc
High K+
25 pA
1 min
10 000 fC
1 min


B
Frequency (Events/min)
50
40
30
20
10
0
**
5 mM gluc
0 gluc


C
Quantal Charge (fC)
40
30
20
10
0
***
5 mM gluc
0 gluc


D
Secretion Rate (fC/min)
4000
3000
2000
1000
0
**
5 mM gluc
0 gluc

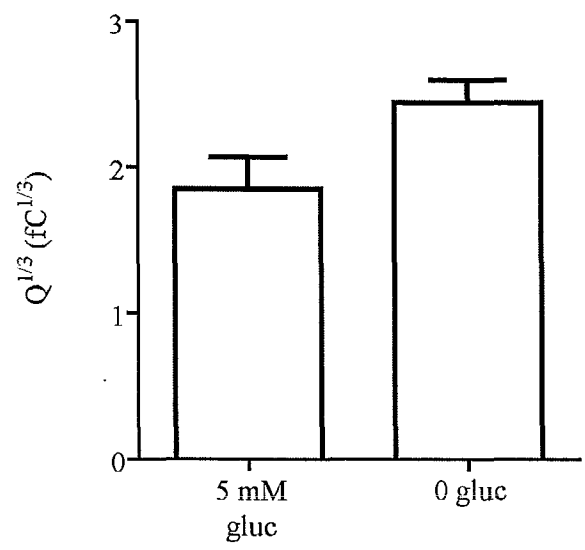
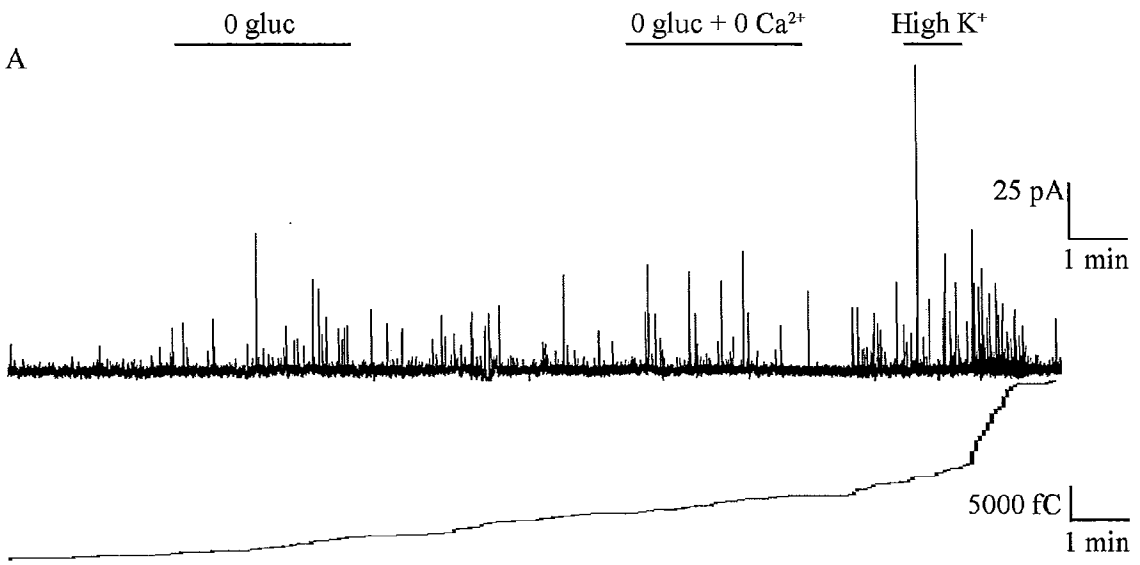
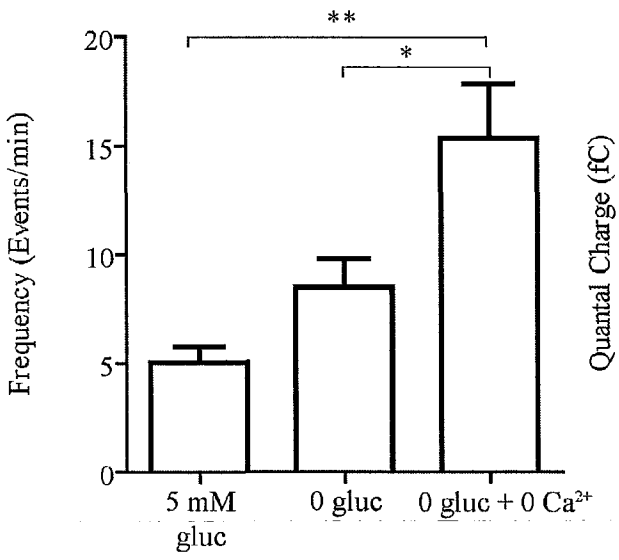
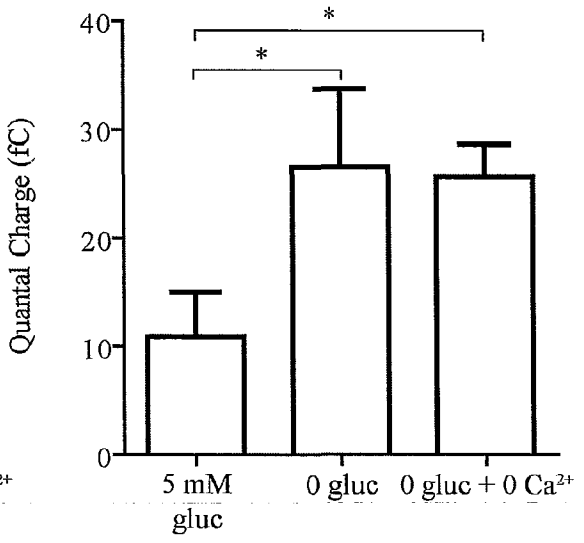
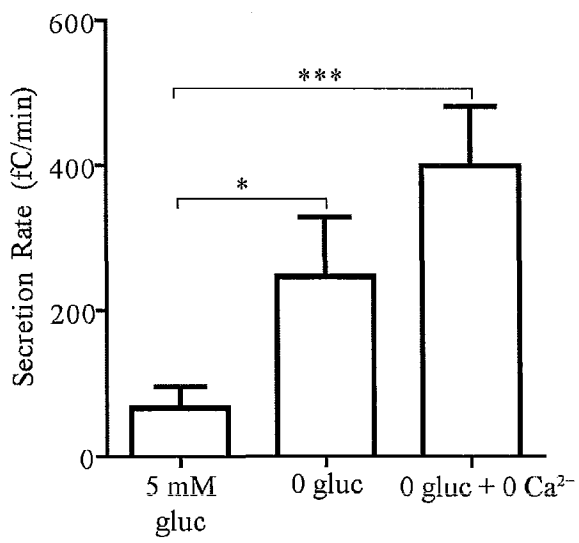

E
Q1/3 (fC1/3)
3
2
1
0
5 mM gluc
0 gluc

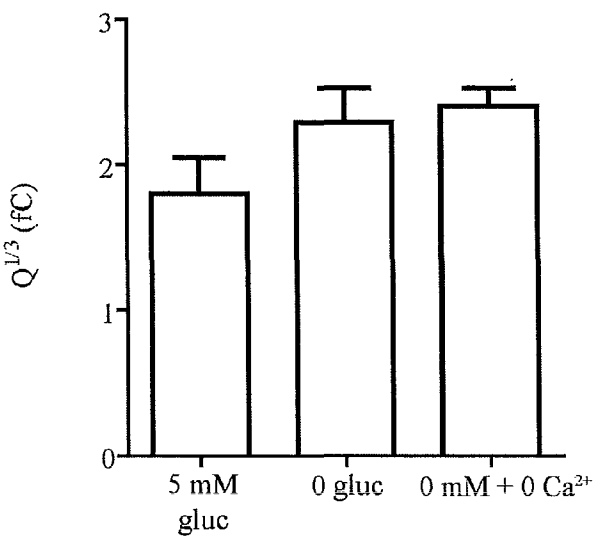
Figure I.2. The aglycemia-evoked secretory response in juvenile AMCs does not require extracellular Ca^{2+}. A, *upper*: trace of a juvenile AMC with aglycemia-evoked secretion in the absence of extracellular Ca^{2+}, *lower*: cumulative charge for the cell above. B, a large increase in frequency in response to aglycemia even in the absence of Ca^{2+}. C, quantal charge was not different when Ca^{2+} was omitted during aglycemia. D, there was robust catecholamine secretion in response to aglycemia when Ca^{2+} was omitted. E, no statistically significant differences in $Q^{1/3}$ was measured. Data represent mean + S.E.M. n = 6 cells, data compared using 1-way ANOVA.


A
0 gluc
0 gluc + 0 Ca2+
High K+
25 pA
1 min
5000 fC
1 min


B
Frequency (Events/min)
20
15
10
5
0
**
*
5 mM gluc
0 gluc
0 gluc + 0 Ca2+


C
Quantal Charge (fC)
40
30
20
10
0
*
*
5 mM gluc
0 gluc
0 gluc + 0 Ca2+


D
Secretion Rate (fC/min)
600
400
200
0
***
*
5 mM gluc
0 gluc
0 gluc + 0 Ca2-


E
Q1/3 (fC)
3
2
1
0
5 mM gluc
0 gluc
0 mM + 0 Ca2+

APPENDIX II

Adrenal slice preparation

SUMMARY

A preparation of fresh (1-8 hours), viable thin slices (~200 μm thick) of the neonatal rat adrenal gland was developed to facilitate future studies of stimulus-evoked catecholamine secretion, without the confounding variables or potential artifacts associated with dissociated cell culture (see Chapters 2 and 3). Whereas juvenile (~2 week-old) adrenal glands proved easy to section, producing healthy slices with robust high K^+-evoked secretion, the small size of neonatal adrenals made this procedure more difficult. A different technique was developed for neonatal adrenal glands and it was possible to measure high K^+-evoked and aglycemia-evoked catecholamine secretion from single cells in some cases.

INTRODUCTION

The use of brain slices began in the 1970s for studies of neurophysiological processes and has become widespread thanks to advances in video microscopy and patch pipettes (Reid *et al.*, 1988). Barbara *et al* (1998) established a thin slice (200-300 μm) preparation to study the cholinergic innervation of adrenomedullary chromaffin cells (AMCs) which was later adapted for studying hypoxia- and hypercapnia-evoked secretory responses in neonatal adrenal slices (Munoz-Cabello *et al.*, 2005; García-Fernández *et al.*, 2007a). Moreover, a similar method has been used to study chemosensory responses in the related carotid body (Pardal & Lopez-Barneo, 2002; García-Fernández *et al.*, 2007b). Adrenal slices offer several advantages over cultured, dissociated AMCs. First, the integrity of the tissue is relatively intact, including gap junctions

and clusters of AMCs. This plays a critical role given the host of paracrine transmitters released by chromaffin cells. Second, they can be used acutely (within an hour of dissection) which limits phenotypic changes which are known to occur in cultured cells. Third, the yield is generally better from slices, resulting in the need for fewer animals to perform the experiments. The principal drawback is that technically it's far more difficult to measure catecholamine secretion from AMCs in slices. There is a great deal of connective tissue as well as damaged tissue along the cutting surface resulting in poor visibility. Moreover, it requires the use of an upright microscope with long water immersion objectives which makes difficult the positioning of the electrodes at the surface of identifiable cells within the slice.

Although not much is known about the drawbacks of adrenal slices, several problems have been identified in brain slices. For instance, metabolic changes occur in brain slices, resulting in lower ATP and phosphocreatine concentrations, less total adenylate, three times more lactate, and higher intracellular pH (Whittingham *et al.*, 1984). Moreover, the brain becomes ischemic during the period between removal from the animal and the completion of the slicing procedure. Reducing PO_2, decreasing glucose levels, or prolonging this period blunts evoked potentials (Whittingham *et al.*, 1984; Schurr *et al.*, 1989). Even after slicing this problem persists because O_2 must diffuse into the slice and, as a result, PO_2 falls abruptly with slice depth. For example, in 400-600 μm thick slices kept in air-equilibrated saline (PO_2 = 150 mmHg) the PO_2 falls to 30 mmHg at about 25 μm below the slice surface and to 1 mmHg at a depth of 150 μm (Bingmann & Kolde, 1982). Moreover, this effect is more pronounced at higher temperatures and in thicker slices. Lastly, brain slices are not immune from phenotypic changes. The selective loss of AMPA receptor subunits, induction of several transcription factors, and changed mRNA expression have been reported in brain slices (Taubenfeld *et al.*, 2002).

MATERIALS AND METHODS

Preparation

The first step was the preparation of the following solutions: (i) Tyrode's solution (in mM): 148 NaCl; 2 KCl; 3 $MgCl_2$; 10 HEPES; 10 glucose; pH 7.4); (ii) bicarbonate-buffered saline (BBS) (in mM): 24 $NaHCO_3$; 115 NaCl; 10 glucose; 12 sucrose; 5 KCl; 2 $CaCl_2$; 1 $MgCl_2$); and (iii) phosphate-buffered saline (PBS) (in mM): 137 NaCl; 2.7 KCl; 8.1 $Na_2HPO_4 \cdot 2H_2O$; 1.76 KH_2PO_4; pH 7.2). Fresh 20 mL solution of 3% agarose (Bioshop, AGA001.500) in PBS was prepared prior to each experiment and brought to a boil. This hot agarose solution was drawn into several 3 mL syringes and then a 16 gauge needle was attached. Syringes were placed in a 46°C water bath and allowed to cool. Tyrode's solution was placed on ice and bubbled with 100% O_2.

Animals

Animal care and handling was performed as described in Chapter 2, however, to limit ischemia after adrenal gland removal a single animal was sacrificed at a time and adrenal slices were taken before proceeding to the next animal.

Juvenile Slice Procedure

Juvenile adrenal gland slicing was done as previously described (Barbara *et al.*, 1998). Adrenal glands were removed and immediately placed in oxygenated Tyrode's solution prior to sectioning. Adrenal glands were glued to the vibratome (Leica Microsystems, Wetzlar Germany) dish with cyanoacrylate, placed in the vibratome tray, and finally covered in oxygenated Tyrode's solution. The razor blade was mounted parallel to the vibratome dish and

200 μm sections were cut at high frequency (setting 10) and low speed (setting ~0.5) settings. The slices were removed from the tray and placed in the wells of a 24-well Falcon plate (Beckinson Dickinson) containing BBS. This plate was kept in a humidified incubator with a 95% air/5% CO_2 atmosphere at 37°C for up to 8 hours.

Neonatal Slice Procedure

Neonatal adrenal glands became embedded in even a single drop of cyanoacrylate; therefore a different method had to be used to obtain slices. Adrenals were removed and placed in oxygenated Tyrode's solution on ice. Using a syringe approximately 0.5 mL of agarose (at 46°) was placed on the vibratome dish. A single adrenal gland was placed into this agarose solution and swirled around. This was done quickly to ensure the gland was included in the agarose block. Once the block hardened (15-30 seconds) extraneous agarose was cut away and the slicing was done as described for the juveniles.

Troubleshooting and alternative procedures

Slices prepared in gelatin (25%, Sigma) were not healthy based on their weak high K^+ responses. Slices included in low melt agarose (4%, Sigma) were difficult to cut and easily came out the block. Neonatal adrenal glands could be glued directly to the Teflon dish with cyanoacrylate; however they became enveloped in the glue. This made slicing more difficult but I did not observe a difference in high K^+-evoked responses. Cutting the slices at faster speeds or lower frequencies pushed the adrenal gland out of the agarose block rather than cutting it. If the razor blade is not parallel to the tray the adrenal gland may be pulled out of the agarose block when the razor blade retracts. Slices could be kept healthy in a hyperoxic (95% O_2/5% CO_2)

atmosphere after cutting; however I could not measure detectable aglycemia-evoked responses from these slices. Hyperoxia can lead to reactive oxygen species (ROS) production which could damage the cells (Chandel & Budinger, 2007). It is often used in brain slices because there is a steep PO_2 gradient, however given that I am recording from AMCs at the surface of the slice this probably is not required.

Imaging Slices

A 22 x 40 mm glass slide was attached to the bottom of the perfusion chamber (RC-22; Warner Instruments) with a thin layer of vacuum grease (Dow Corning). This was then attached to a slice platform (P-1; Warner Instruments) with vacuum grease and then held in place with side clamps. Slices were placed in the slice chamber and pinned to the slide with a slice anchor (SHD-22L/15; Warner Instruments) made of a stainless steel harp with Lycra cross threads. The slice platform was placed on the stage of an Axioskop upright microscope (Zeiss) equipped with long distance water immersion objectives. Slices were viewed with differential interference contrast microscopy and imaged with infrared video microscopy (XC-73, Sony) on a closed-circuit television (CPT-CM 15, Capture, Korea). Pictures of the slices were taken by removing the infrared camera and replacing it with a QICAM Fast camera (QIMAGING) attached via a fire-wire cable to a computer. Slices were visualized using QCapture software (QIMAGING) by opening the "Live Preview" option. Settings could be manipulated (e.g. contrast, exposure.) and a picture taken by pressing "Snap". Unfortunately, with the infrared camera removed resolution of the adrenal slices was poor.

Amperometry

Amperometry was performed as previously described (Chapter 2), however, there were slight modifications required for slice work. The carbon fibre electrode was placed in the medulla of the slice in close proximity to a cluster of cells. The slice was perfused with a high K^+ solution and healthy slices responded with a large upward shift in the baseline indicating a rise in bath catecholamines. This was repeated until quantal catecholamine release was measured from AMCs and then the slice was perfused with aglycemia to test for chemosensitivity.

RESULTS

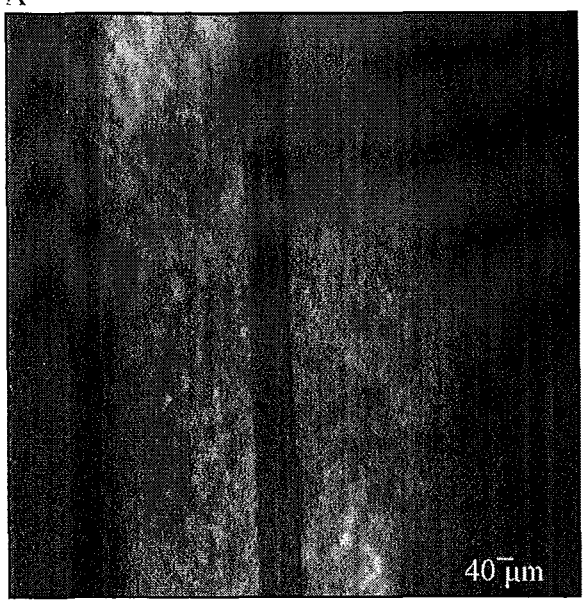
The adrenal medulla region was clearly identifiable in juvenile and neonatal slices as a transparent region in the centre of the adrenal gland (Fig II.1A, C). At higher magnification, some clusters of AMCs could be identified in juvenile slices (arrows) (Fig II.1B). However, in neonatal slices individual cells or clusters were more difficult to observe (Fig II.1D). In amperometric recordings a slow upward shift in the baseline during high K^+ exposure corresponded to an increase in bath catecholamines and was used to determine whether or not slices were healthy (Fig II.2A-E). When the carbon fiber microelectrode was close to juvenile AMCs quantal catecholamine secretion could be measured (Fig II.2A, B). This phenomenon was also observed in neonatal AMCs (Fig II.2C-D). Some neonatal AMCs in slices were not sensitive to aglycemia (Fig II.2C-D). Occasionally, a rise in baseline was observed in response to aglycemia in neonatal slices (Fig II.2D), and in some cells quantal catecholamine secretion was measured in response to aglycemia (Fig II.2E).

This secretory response was characterized by an increase in the frequency of quantal events (Fig II.3A, $p<0.05$, $n = 3$). There was no significant change in mean quantal charge or $Q^{1/3}$ in slices, in contrast to the effects in cultured AMCs (Chapter 2) (Fig II.3B, D). Overall, there was an increase in secretion rate in response to aglycemia in neonatal adrenal slices (Fig II.3C, $p<0.05$, $n = 3$) and the magnitude of the responses was similar to that observed in cultured cells.

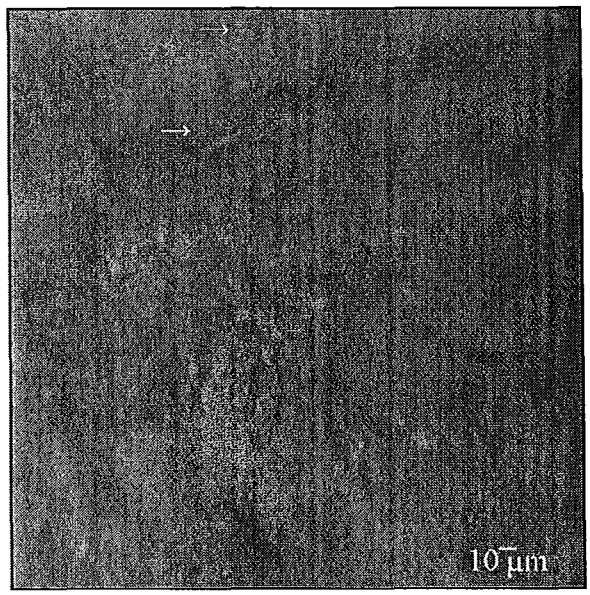
Figure II.1. Images of juvenile and neonatal rat adrenal slices. A, low magnification image of adrenal gland showing the translucent adrenal medulla, bounded by the darker adrenal cortex; a Lycra cross thread can be identified pinning the slice to the slide. B, higher magnification image of juvenile adrenal medulla showing a couple clusters of AMCs (arrows). C, image of neonatal adrenal gland with clear medulla and cortex. D, high magnification image of neonatal adrenal medulla showing poor visibility of AMCs. These images were taken with DIC microscopy without infrared microscopy.

Juveniles

A

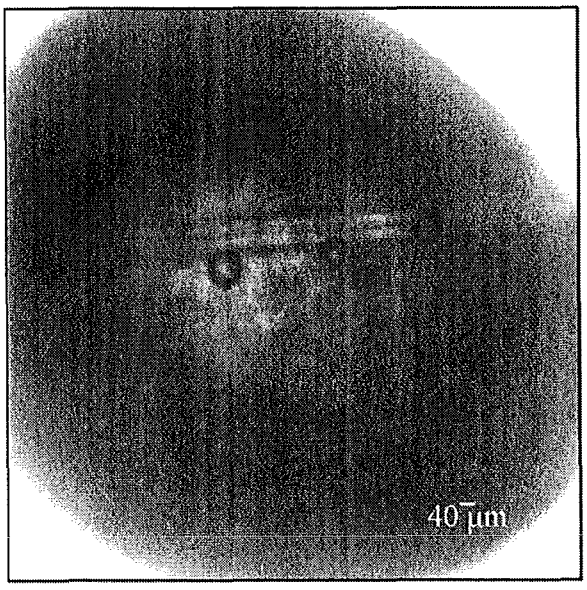


B



Neonates

C



D

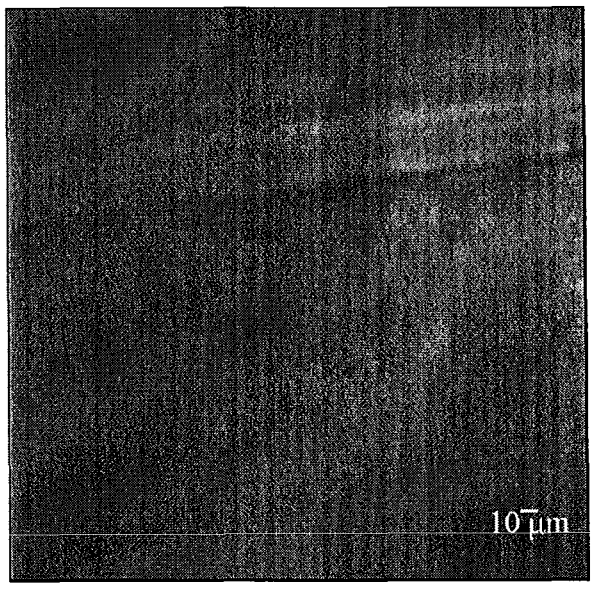
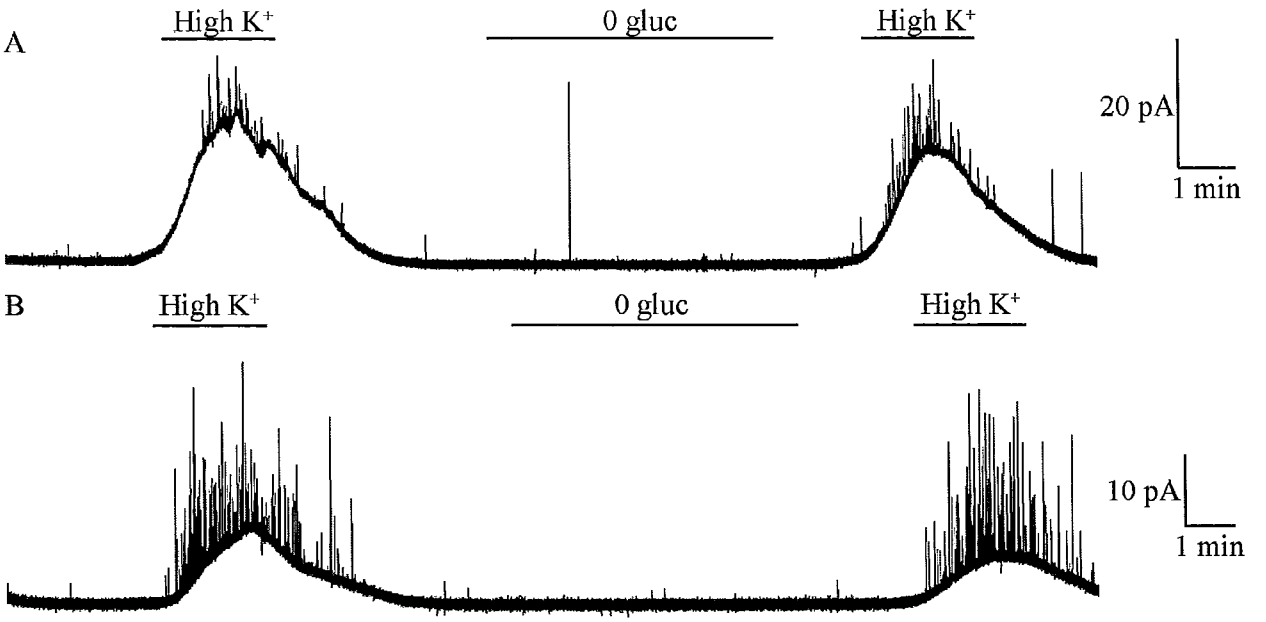


Figure II.2. Traces of responses observed from juvenile and neonatal AMCs in slices. A, B, the large upward shift in the baseline during high K^+ exposure indicates an increase in bath catecholamines which occurs in parallel with quantal catecholamine release in juvenile slices. C, a recording from a neonatal adrenal slice with high K^+-evoked secretion but no aglycemia-evoked secretion. D, a trace showing a rise in bath catecholamines during aglycemia, but no quantal release in AMCs from a neonatal slice. E, exemplar trace from a neonatal adrenal slice showing quantal release in response to aglycemia.

Juveniles



Neonates

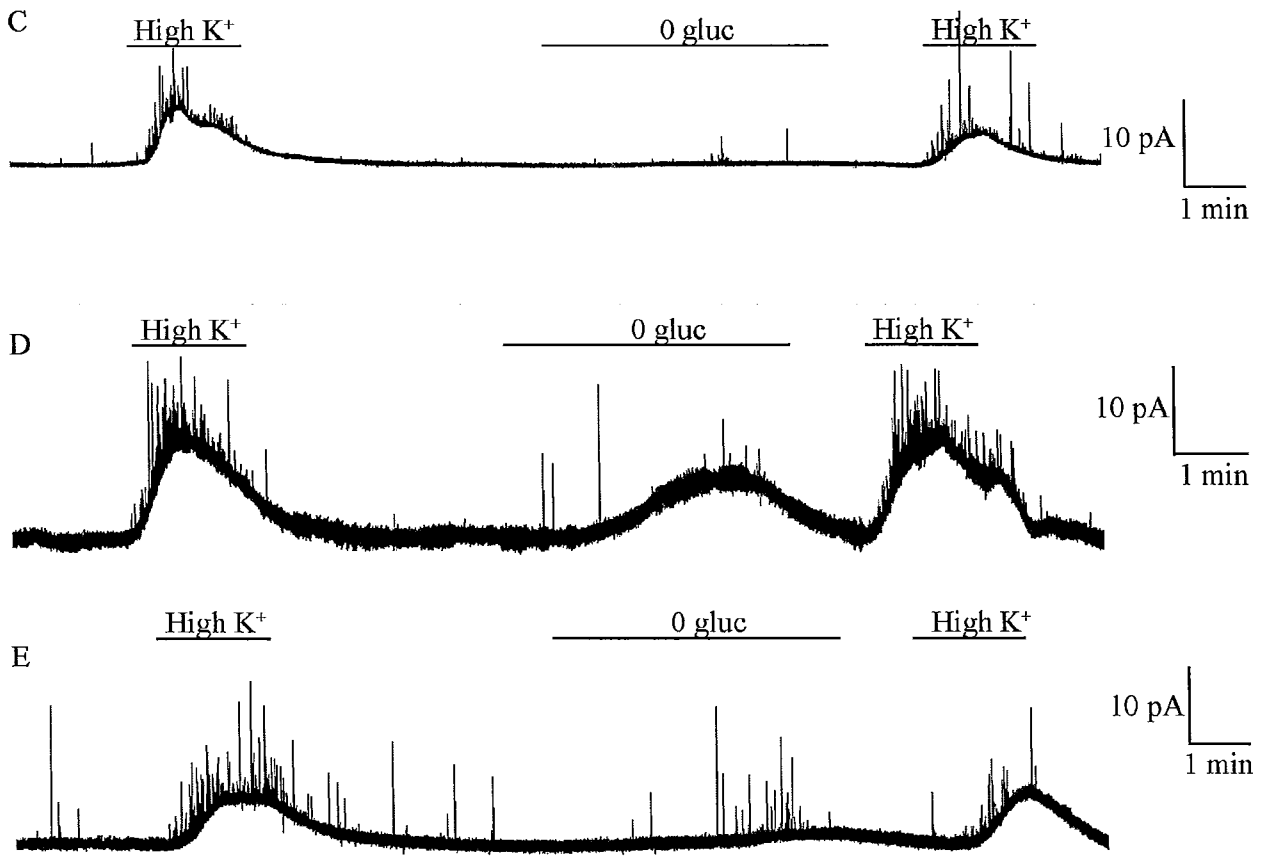
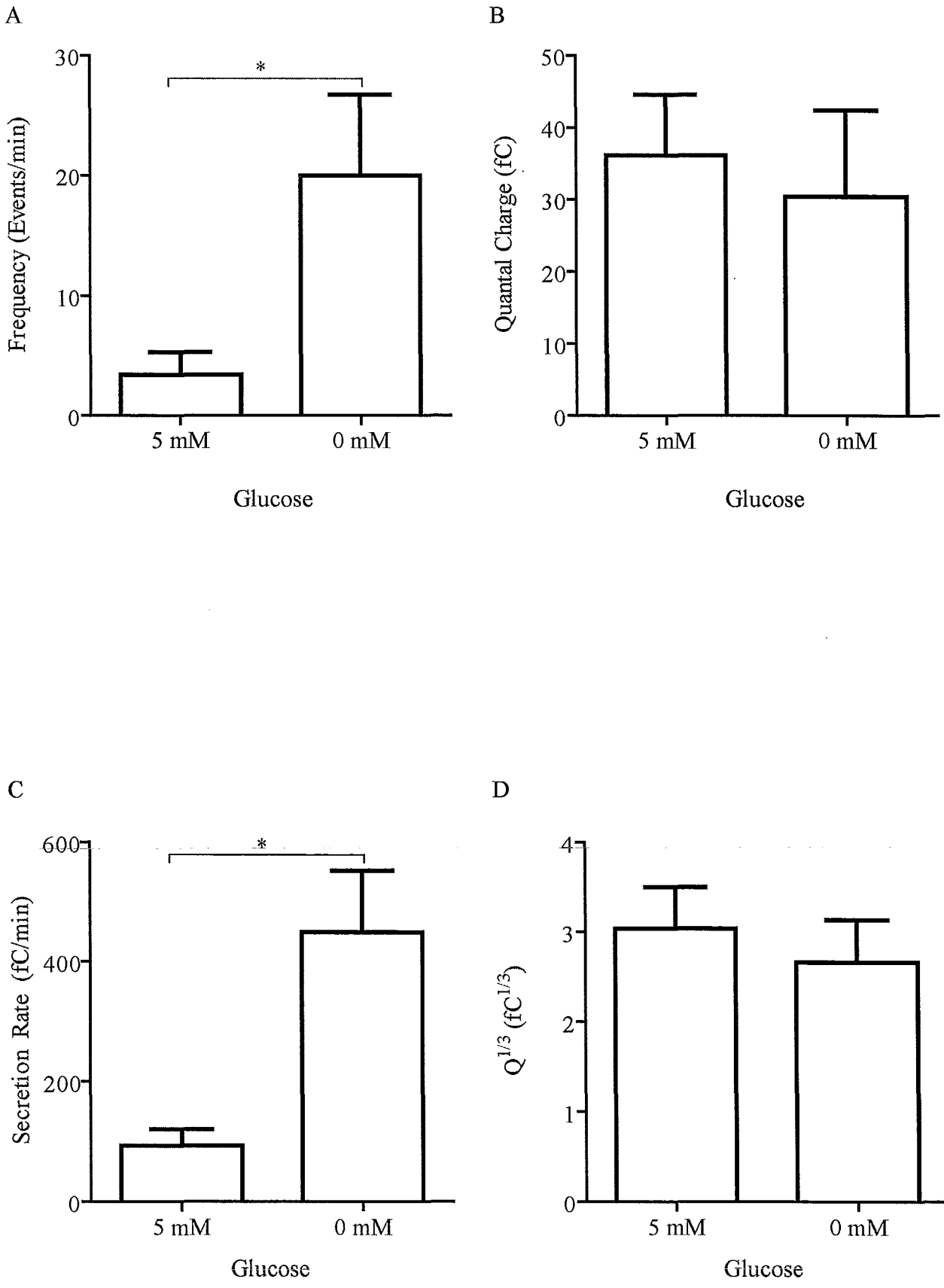


Figure II.3. Characterization of secretory responses to aglycemia in a few neonatal adrenal slices. A, in a few cells there was an increase in the frequency of quantal events in response to aglycemia. B, there was no significant change in quantal charge during aglycemia. C, aglycemia produced a large increase in secretion rate. D, there was no significant change in $Q^{1/3}$ during aglycemia. Data represent mean ± S.E.M. (n =3 AMCs), and were compared using a one tailed Student's t-test.


A
B
C
D
Frequency (Events/min)
Quantal Charge (fC)
Secretion Rate (fC/min)
$Q^{1/3}$ ($fC^{1/3}$)
5 mM
0 mM
Glucose
*

APPENDIX III

Attempts to measure ATP release from neonatal adrenomedullary chromaffin cells

SUMMARY

I attempted to measure stimulus-evoked ATP secretion from neonatal AMCs, juvenile adrenal slices, and whole neonatal adrenal glands. Using a protocol which has been used previously in this lab, I was unable to measure stimulus-evoked ATP secretion. I developed a novel method of measuring ATP, however, there was no convincing demonstration that ATP release occurred in these preparations.

INTRODUCTION

Neonatal AMCs respond to hypoglycemia via voltage-gated Ca^{2+} entry and the fusion of catecholamine vesicles with the plasma membrane (Chapter 2). These catecholamines are co-stored along with ATP in dense core vesicles in a 4:1 ratio (Winkler & Westhead, 1980). ATP can act as an autocrine/paracrine modulator via cell membrane receptors before being broken down by ecto-ATPases into ADP. ATP, along with other purine di- and trinucleotides, activates ionotropic P2X receptors and metabotropic P2Y receptors. ATP release from chromaffin cells has been previously measured and is known to modulate many intracellular signalling pathways in AMCs (Rojas *et al.*, 1985; Kasai *et al.*, 1999). In this lab, a technique has been developed to measure ATP release from whole carotid bodies, carotid body thin slices, and dispersed carotid body glomus cells (Buttigieg & Nurse, 2004; Zhang *et al.*, 2007). Moreover, it has also been used to measure ATP release from AMCs (Buttigieg *et al.*, 2006; Thompson *et al.*, 2007). This technique utilizes the bioluminescence reaction catalyzed by the firefly enzyme luciferase to measure ATP release via the following reaction:

$$\text{luciferin} + \text{ATP} \rightarrow \text{luciferyl adenylate} + PP_i$$

$$\text{luciferyl adenylate} + O_2 \rightarrow \text{oxyluciferin} + \text{AMP} + \text{light}$$

The firefly enzyme luciferase catalyzes the reaction of ATP and luciferin to form luciferyl adenylate and inorganic phosphate. The oxidation of luciferyl adenylate produces oxyluciferin, AMP, and a photon of light. A photomultiplier tube captures these photons and converts them into a digital signal. This signal is read by a luminometer and is expressed as relative light units (RLU). When ATP is the limiting reagent, this reaction can be used to measure its concentration. Using this technique, I attempted to measure stimulus-evoked ATP release from neonatal AMCs in culture, in fresh tissue slices, and whole adrenal glands.

MATERIALS AND METHODS

Animal Procedures

Animal handling was done as explained in Chapter 2.

Cell Culture Method

Preparation of neonatal AMC cell culture was done as described in Chapter 2, with slight modifications. Instead of culturing cells in a dish they were cultured in a modified luminometer plate. The modified plate was constructed by painting glass rings white and attaching them to the lid of a 24-well luminometer plate (Falcon 35047) with sylgard (Paisley Products, Mississauga). These dishes were allowed to dry then they were placed under UV light for 2 hours. AMCs were cultured on the bottom of these cells on top of a layer of matrigel (Becton Dickenson). The cells were then placed in a humidified atmosphere of 95% air/5% CO_2 for 24 hours before experiments were performed.

Adrenal Slices

Adrenal slices were prepared as described in Appendix II.

ATP Assay

Two protocols were used to measure stimulus-evoked ATP release. Protocol 1 was previously used in the lab to measure ATP secretion from dispersed carotid body glomus cells and AMCs (Buttigieg & Nurse, 2004; Buttigieg *et al.*, 2006). Because I had difficulty measuring stimulus-evoked ATP release using this method (results below), I experimented with a new method, Protocol 2, in an attempt to address some of the drawbacks of Protocol 1.

Protocol 1: Experiments were performed on neonatal AMCs cultured overnight in a modified luminometer dish, freshly dissected whole adrenal glands, and fresh tissue slices. Cells were plated into a luminometer well with 800 µL HEPES-buffered saline solution. A 10 mL stock solution of luciferin-luciferase solution (ATP determination kit; Molecular Probes #A22066) was made and kept in the dark on ice. This solution contained:

- 8.9 mL dH20
- 0.5 mL 20x Reaction Buffer (contains 500 mM Tricine buffer, pH 7.8, 100 mM $MgSO_4$, 2 mM EDTA, 2 mM NaN_3)
- 0.5 mL of 10 mM D-luciferin
- 2.5 µL luciferase

200 µL of this stock solution was added to a well containing the AMCs at room temperature and then the luminometer dish was placed into a Labsystem Luminoskan Luminometer. The emitted

photons were read as RLU and collected and stored using Labsystems Ascent Software. RLU were converted to concentration of ATP ([ATP]) using a standard curve.

Protocol 2: Because ATP release could not be measured in response to stimuli that evoked catecholamine release, several different potential problems were identified with Protocol 1, and a new protocol was designed to avoid these problems.

1) Adding the cold stock solution (kept on ice) to the cells during the experiment may produce cell stress and lead to cell death.
2) The maximum enzymatic reaction for luciferase occurs at pH = 7.8. At the pH of the previous protocol (7.4) activity would be reduced. Alternatively, adding the stock solution at pH 7.8 could stress the cells and may contribute to cell death.
3) The luciferin-luciferase stock solution contains NaN_3, which inhibits cytochrome oxidase (Stannard & Horecker, 1948) and is known to cause cell death (Nicoletti *et al.*, 1991).
4) The reaction mixture contains 100 μM EDTA which is often used to resuspend cells and could lead to cell lysis.
5) The protocol suggested by Invitrogen recommends no more than 10% sample size and 90% reaction mixture, as opposed to the 80% sample size and 20% reaction mixture previously used. There are a number of reasons for ensuring a small sample size to reaction mixture ratio:
    a. Ensure that ATP is the limiting reagent in the reaction so that this is what is being measured.

b. Mg^{2+} is a cofactor for luciferase, and is suggested to be at a final concentration of 5 mM, instead of the value closer to 2 mM in the previous protocol.

In order to avoid these potential complications, a new protocol was established. Two adrenal tissue slices or a single adrenal gland was placed in a 100 µL eppendorf tube containing HEPES-buffered saline at room temperature. The tissue was incubated in the tube for 15 minutes and then a 10 µL sample was removed and placed in a well of a 96 well luminometer plate (Falcon #353219). 90 µL of stock luciferin-luciferase reaction mixture was added to the sample and the plate was placed in the luminometer and RLU was recorded for 5 minutes. The control solution was replaced with a high K^+ (30 mM KCl) solution and left for 15 minutes before a 10 µL sample was taken and RLU was measured as before. This protocol has three principal advantages, i.e. cells are not exposed to EDTA, rapid changes in temperature, nor NaN_3. Critically, it also ensures maximal luciferase activity, correct pH, right buffer conditions and high Mg^{2+} concentrations. The principal drawback is that ATP release is not measured in real time.

Solutions:

The solutions used in the experiments presented are identical to those used in Chapter 2.

RESULTS

Using Protocol 1, ATP release was measured from dispersed AMCs. It was found that exposure of cells to hypoxia increased ATP release; however the cells did not recover after this stimulus (Fig III.1A). Similarly, in response to aglycemia, there was a rise in ATP; however no

recovery was observed (Fig III.1B). Critically, significantly fewer cells were found on the plate during each successive stimulus. I hypothesized that ATP release could be occurring as a result of cell death during successive solution changes rather than secretion. Therefore, I made successive changes of control saline with identical control saline and monitored ATP release. As shown Figure III.1C successive saline changes increased the concentration of ATP in a manner similar to hypoxia. Because changing the solution may cause cells to detach from the bottom of the plate, I used thin slices of adrenal glands and whole adrenal glands and measured ATP release. Thin slices of juvenile adrenal glands were prepared and exposed to 15 minutes of normoxia followed by 15 minutes of high K^+ (Fig III.1D). Although a large concentration of ATP was measured, there was no difference between control solution and high K^+ stimulation. Similarly, whole neonatal adrenal glands were exposed to 15 minutes of control solution followed by 15 minutes of high K^+ and no difference was observed in the concentration of ATP measured (Fig III.1E).

I therefore identified problems with the previous protocol and established Protocol 2. Comparison of the standard curves obtained using both protocols shows that they are both sensitive to ATP, with protocol 2 being slight more sensitive (Fig III.2A). Juvenile adrenal slices showed no change in ATP in response to aglycemia or high K^+, when incubated for 15 minutes in each (Fig III.2B) and neither did whole adrenal glands (Fig III.2C). It is possible that ATP levels had not increased because ATP was being broken down by ecto-ATPases. The ecto-ATPase inhibitor FPL 67156 (100 μM) was incubated with cells in high K^+ for 15 minutes and no change in ATP was observed (Fig III.2D). Note that the concentrations of ATP shown for these experiments (~2 nM) are below the resolving power of the luminometer and therefore should be considered ~0 nM.

Figure III.1. ATP release from cultured neonatal AMCs, juvenile slices, and whole neonatal adrenal glands. A, hypoxia increased ATP release, however there was no recovery after washout; high K^+ (30 mM KCl) had a weak effect on ATP (n = 5 wells). B, aglycemia increased ATP levels in the extracellular solution (n = 6 wells). C, successive washing of the cells with control solution increased ATP in a manner similar to hypoxia (n = 4 wells). D, juvenile thin slices glands cultured in control or high K^+ solutions for 15 minutes showed no differences in concentrations of ATP (n = 6 wells). E, there was no significant difference in high K^+ and control (basal) ATP release in whole neonatal adrenal glands (n = 8 wells).

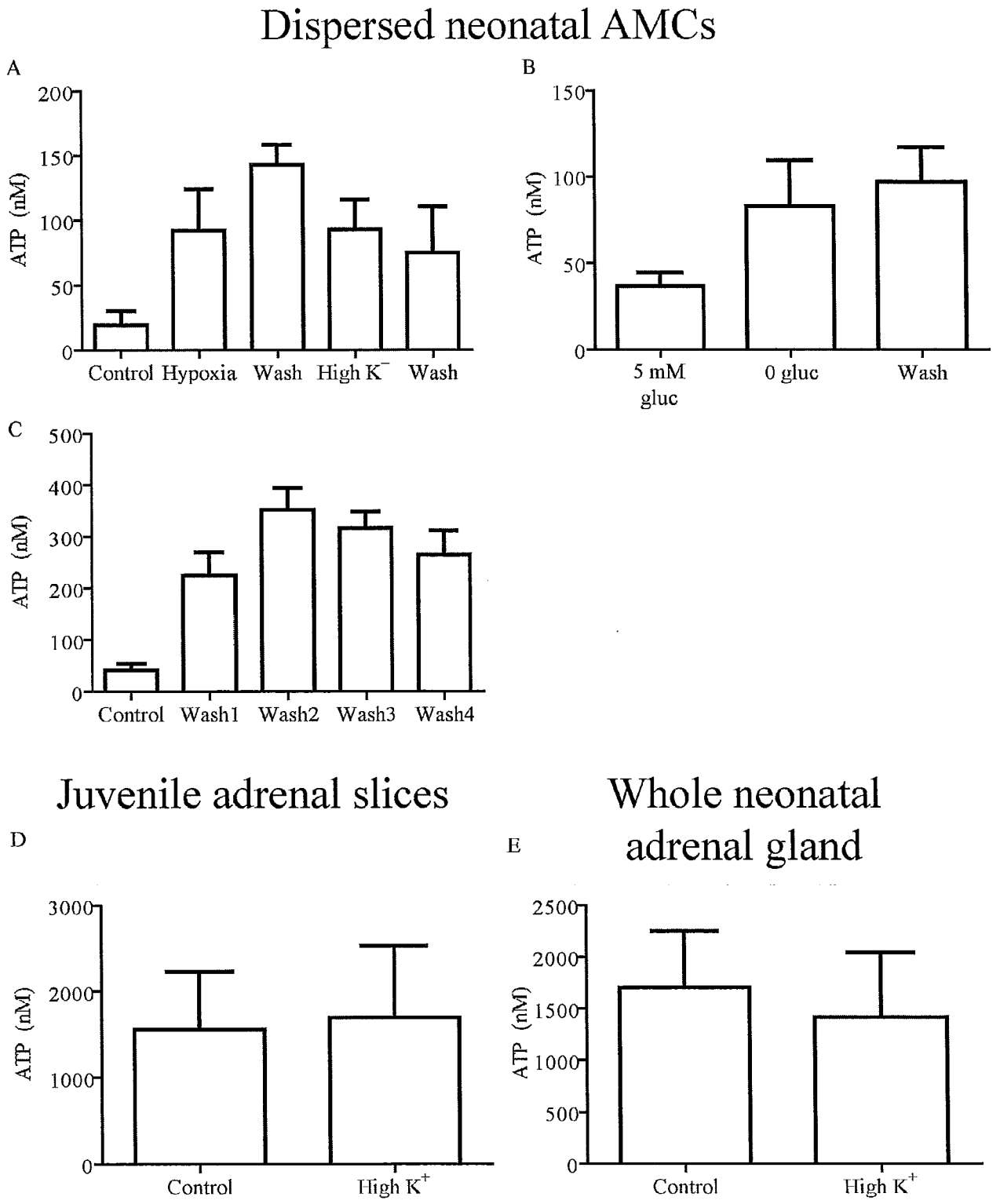
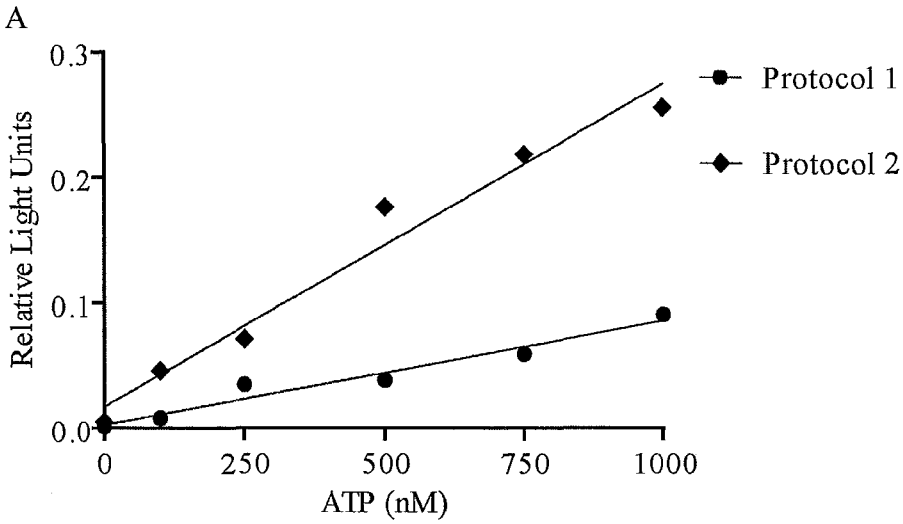
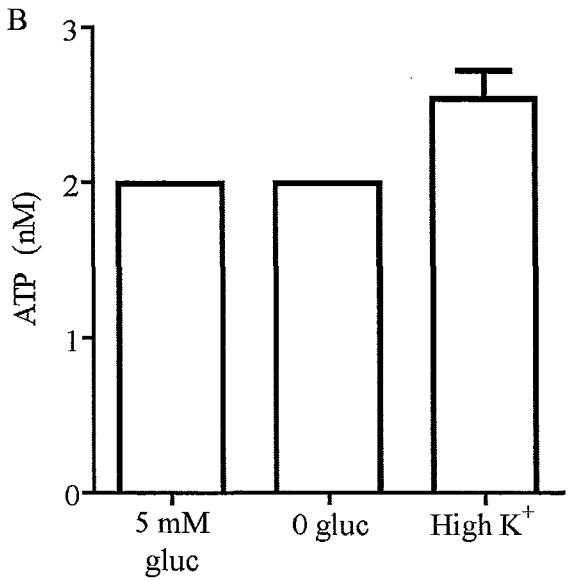
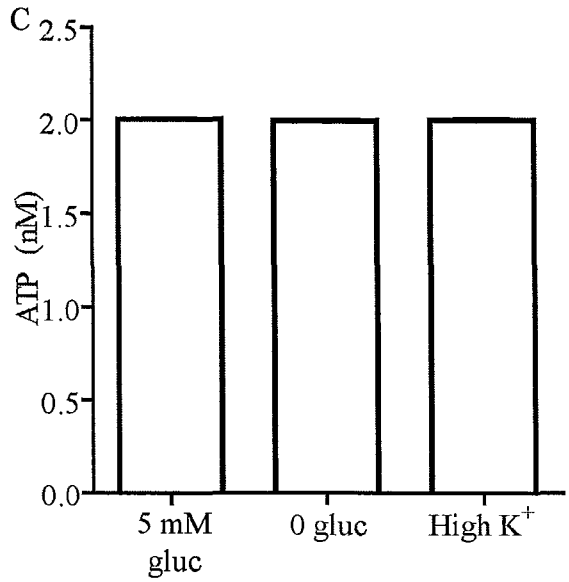
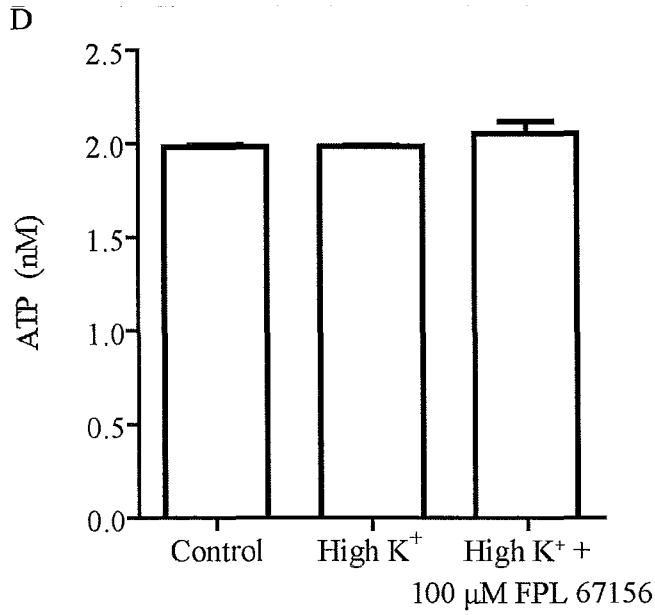

Dispersed neonatal AMCs
A
ATP (nM)
200
150
100
50
0
Control
Hypoxia
Wash
High K⁻
Wash
B
ATP (nM)
150
100
50
0
5 mM gluc
0 gluc
Wash
C
ATP (nM)
500
400
300
200
100
0
Control
Wash1
Wash2
Wash3
Wash4
Juvenile adrenal slices
D
ATP (nM)
3000
2000
1000
0
Control
High K+
Whole neonatal adrenal gland
E
ATP (nM)
2500
2000
1500
1000
500
0
Control
High K+

Figure III.2. Changes in ATP measured by Protocol 2. A, comparison of assay sensitivity using Protocols 1 and 2. B, juvenile adrenal slices showed no measurable change in ATP in response to any stimulus (n = 6 wells). C, whole neonatal adrenal glands showed no change in ATP levels in response to aglycemia or high K^+ (n = 3 wells). D, the ecto-ATPase inhibitor FPL 67156 had no effect on ATP concentration in high K^+ in juvenile adrenal slices (n = 4 wells).


A
0.3
0.2
0.1
0.0
Relative Light Units
0
250
500
750
1000
ATP (nM)
Protocol 1
Protocol 2


B
3
2
1
0
ATP (nM)
5 mM gluc
0 gluc
High K+


C
2.5
2.0
1.5
1.0
0.5
0.0
ATP (nM)
5 mM gluc
0 gluc
High K+


D
2.5
2.0
1.5
1.0
0.5
0.0
ATP (nM)
Control
High K+
High K+ +
100 µM FPL 67156

APPENDIX IV

Composition of Solutions

L-15 Plating Medium
0.5 mL penstrep + 0.5 mL glutamine

0.5 mL 30% glucose

Make up to 50 mL with L-15 Stock

Enzyme

5 mL of 10x Hanks BSS (minus Ca^{2+}, Mg^{2+}, $NaHCO_3$)

45 mL of cell culture water

59.6 mg HEPES

50 mg Collagenase

50 mg Trypsin

5 mg DNAse I

Mix until dissolved. Adjusted to pH 7.2 with 1 N NaOH

Sterilized by passage through a 0.2 μm filter

Add 0.5 mL Pen-strep

Frozen into 1.5 mL aliquots before being added to adrenal medullae.

AMC F-12 Medium

0.5 mL Pen-strep

0.5 mL 30% glucose

0.5 mL glutamine

0.2 mL insulin

5 mL Fetal Bovine Serum

0.5 mL Dexamethasone

43.5 mL F-12 Medium

MAH Medium

0.5 mL Fresh vitamin mix

2 mL of 1:1:2 (Glucose:Glutamine:Pen-strep)

5 mL Fetal Bovine Serum

0.5 mL Dexamethasone

42 mL L-15/CO_2

AMC DMEM Medium

0.5 mL Pen-strep

0.2 mL insulin

5 mL Fetal Bovine Serum

0.5 mL Dexamethasone

0.5 mL glutamine

43.3 mL DMEM

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