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Maturation modulates both synthesis and degradation of cGMP in ovine vascular smooth muscle

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MATURATION MODULATES BOTH SYNTHESIS

AND DEGRADATION OF cGMP IN OVINE VASCULAR

SMOOTH MUSCLE

A Thesis

Presented to the

Faculty of

California State University,

San Bernardino

In Partial Fulfillment

of the Requirements

for the Degree

Master of Science

in

Biology
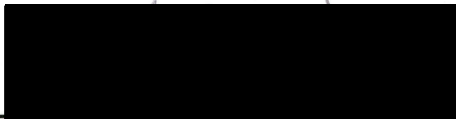
by

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Approved by:

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Date

ABSTRACT

A previous study of cGMP dynamics in the ovine newborn and adult common carotid artery (COM) by Pearce et. al., 1994. showed that the basal cGMP levels in the COM were significantly higher in the newborn compared to the adult (0.508 ± 0.09 and 0.11 ± 0.01 pmol cGMP $\cdot$ L cell water $^{-1}$, respectively) ($P<0.05$). This observation may represent a significant difference in the overall metabolism of cGMP. Furthermore, this difference may be modulated by maturation. Given the central importance of cGMP metabolism in maintaining vascular tone, this difference in basal cGMP may partly explain the high incidence of cerebrovascular complications in the newborn. In order to investigate the mechanisms involved in this phenomenon, this project was designed with two specific aims. The first, to examine and compare the activities of guanylate cyclase (GC) and phosphodiesterase (PDE) (*the enzymes responsible for cGMP synthesis and degradation*) in newborn and adult sheep common carotid arteries. The second, to determine if intracellular compartmentalization plays a role in PDE activity. To examine the first question, I developed a novel technique to simultaneously estimate rates of synthesis and degradation of cGMP in whole, unbroken arteries. The estimated synthetic rate for cGMP in the newborn was higher compared to the adult (7.62 ± 1.14 and 5.94 ± 1.26 pmol cGMP $\cdot$ L cell water $^{-1} \cdot$ min $^{-1}$, respectively). Similarly, the estimated rate of cGMP degradation was also higher in the newborn compared to the adult ($4.26 \pm .84$ and $2.88 \pm .78$ pmol cGMP $\cdot$ L cell water $^{-1} \cdot$ min $^{-1}$). Although the synthetic and degradative rates for cGMP were different between the two age groups, the net difference between cGMP synthesis - degradation was similar in both the newborn and adult ($3.36 \pm .6$ and $3.06 \pm .6$ pmol / L cell

water / min, respectively). These results indicate that the difference between synthesis - degradation is similar in both newborn and adult, and it is the absolute levels of cGMP which differ. In order to investigate the second question, I compared the estimated rates of cGMP degradation for each age group in the whole-unbroken arteries with rates determined for each age group in broken cell preparations. I utilized a crude homogenate approach where the rate of cGMP substrate disappearance was used as an index of PDE activity. In the crude homogenate preparation, the rate of cGMP degradation was higher in the adult compared to the newborn ($2.406 \pm .224$ and $1.407 \pm .157$ pmol cGMP $\cdot$ L cell water $^{-1} \cdot$ min $^{-1}$, respectively). This relationship between the newborn and the adult is in contrast to the results of the whole-unbroken arteries. These results suggest that intracellular compartmentalization does play a role in PDE activity.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

page

INTRODUCTION

A Brief History and Overview of cGMP Metabolism

Cyclic nucleotides play a variety of roles in the regulation of cellular functions in nearly all cell types. Of these roles, one of the most important, is that of an intracellular secondary messenger within vascular smooth muscle [Lincoln, 1989]. Two of the most abundant cyclic nucleotides are adenosine 3', 5'- cyclic monophosphate (cAMP) and guanosine 3', 5'- cyclic monophosphate (cGMP). cAMP plays an important role in blood vessel relaxation caused by beta-adrenergic agonists. cGMP participates in blood vessel relaxation induced by such drugs as nitroglycerin, nitroprusside and agents that function by stimulating the release of an endothelium derived relaxing factor (EDRF). Although the existence of cGMP was first reported more than 25 years ago, not long after the discovery of cAMP, the role it plays as a secondary messenger remained mostly unexplored for 10 - 12 years [Goy, 1991]. The majority of research conducted into cyclic nucleotides was focused on cAMP. cGMP was thought to be merely an antagonist or analogous to cAMP. Because cAMP was considered to be an important mediator of smooth muscle relaxation, it was then reasoned that cGMP must be a mediator of contraction. It was not until 1977 when several investigators [Katsuki and Murad, 1977] [Katsuki et al., 1977] [Schultz et al., 1977] showed that vasorelaxation induced by nitrovasodilators (nitrogen-oxide constituents) was associated with an increase in cGMP levels that new interest was generated in cGMP [Lincoln, 1989].

Over the last 15 years a more comprehensive picture of cGMP and its mechanisms of vasodilatation has emerged. **Figure 1** is a simplified overview of the biological mechanisms that participate in the cGMP cascade which leads to relaxation in vascular smooth muscle. Under normal
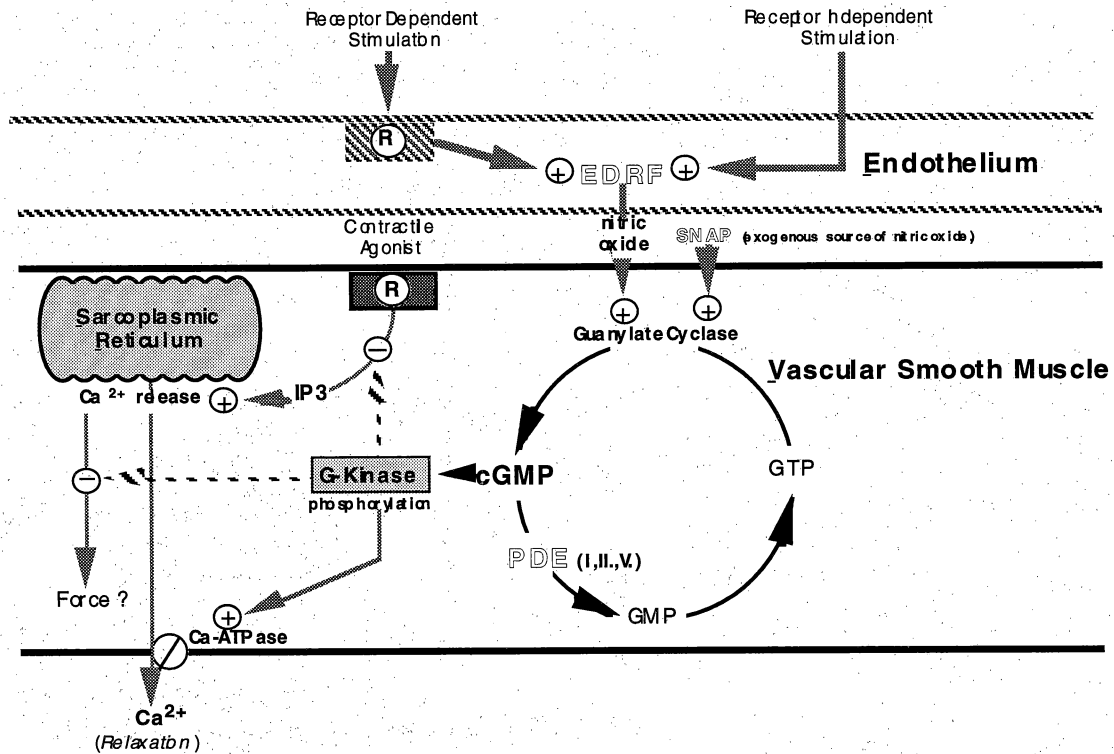
Figure 1: A Basic Overview of cGMP Metabolism and Mechanisms of Vasodilatation.

physiological conditions, the endothelium stimulates vascular smooth muscle relaxation through the release of EDRF. Since its discovery in the early 1980's [Furchgott and Zawadzki, 1980], EDRF has been shown to be essentially nitric oxide [Hardman, 1984] [Ignarro, et al., 1987]. The endothelium can also produce other factors which are capable of inducing relaxation, such as endothelium derived hyperpolarizing factor (EDHF), but these factors are not thought to be involved in the cGMP cascade. Although the endothelium plays

the most important role in the release of nitric oxide into the smooth muscle cells, there are other pathways for nitric oxide to enter the cell. Ignarro has reported the presence of small quantities of NO-synthase in the smooth muscle itself [1992 FASEB meetings, Anaheim CA]. Drugs such as nitroglycerin and nitroprusside do not require the presence of the endothelium, and function via biotransformation within the smooth muscle cell to release nitric oxide [Ignarro et al., 1981] [Brien et al., 1988]. Still other drugs such as S-nitroso-N-acetyl-penacillamine (SNAP) release nitric oxide spontaneously upon hydration to serve as an exogenous source of nitric oxide [Ignarro et al., 1981].

No matter its source, nitric oxide directly stimulates guanylate cyclase (GC) to convert the substrate guanosine triphosphate (GTP), into cGMP. Once GC is activated, cGMP levels quickly begin to rise. cGMP then directly binds with, and thus activates cGMP-dependent protein kinase (G-kinase). Although the mechanics of G-kinase are not fully understood, it is known that once activated, G-kinase acts via phosphorylation of various unknown protein substrates within the smooth muscle cell. It has been proposed that this phosphorylation affects at least three different pathways in order to regulate intracellular levels of Ca^{2+}. It is these pathways, alone or combined, that lead to relaxation. Smooth muscle tone is dependent upon intracellular levels of Ca^{2+}. High levels of Ca^{2+} induce contraction, whereas low levels are associated with relaxation. The first of these pathways leads to the direct reduction of Ca^{2+} within the cell by the activation of Ca-ATPase pumps, which pump Ca^{2+} out of the cell. The second pathway is capable of attenuating the polyphosphoinositide cycle (specifically IP3). This reduces the amount of stimulation by IP3 on the sarcoplasmic reticulum to extrude Ca^{2+}, thus

3

preventing intracellular levels of free Ca^{2+} from rising [Abdel-Latif A. A., 1986].

The third pathway effects the state of force produced by the myosin and actin

filaments of the smooth muscle itself. G-kinase is capable of phosphorylation

at no less than seven sites within the myosin-actin complex, thus altering the

characteristics of the actin filaments which allow them to bind and interact with

myosin. This change in the physical characteristics of actin and myosin (which

would also include its sensitivity to Ca^{2+}) leads to a change in the force

produced by the vascular smooth muscle [Pearce and Harder, 1994].

Ultimately, the magnitude and the duration of relaxation is directly

linked to the amount of phosphorylation by activated G-kinase. If cGMP levels

within the cell were to remain elevated above baseline for an extended period

of time, nearly full and complete activation of G-kinase would occur.

Conditions such as these would render the vascular smooth muscle incapable

of maintaining tone. Therefore, the cGMP signal which controls the activation

of G-kinase should be very short in duration. This is accomplished within the *in

vivo* system by a rapid and short pulse of cGMP. In order to achieve this rapid

and short pulse, not only must rapid synthesis of cGMP by GC occur, but rapid

degradation of cGMP must also occur at roughly the same time. In adult

sheep, the cGMP pulse has been shown to reach peak cGMP concentration by

60 sec. after NO stimulation, and then rapidly return back to baseline by 100

sec. [Pearce, et al., 1994]. The family of enzymes responsible for this rapid

degradation (hydrolysis of the 3'-phosphodiester bond) of cGMP, are known as

cGMP specific phosphodiesterases (PDE). The hydrolysis of cGMP in this

fashion simply yields guanosine monophosphate (GMP) which may then go on

to complete the cycle and return to GTP.

In order for an organism to possess a dynamic vascular system capable of responding quickly and repeatedly to a changing environment, tight control over intercellular levels of cGMP must be maintained. To achieve this, synthesis and degradation of cGMP must be tightly linked together. It is the balance or "ratio" of GC and PDE activity within the dynamic system which determines the total magnitude of the cGMP pulse, which in turn determines the amount of phosphorylation by G-kinase, and which finally determines the magnitude and the duration of vasodilation.

A variety of different research projects and disciplines have lead us to this model of cGMP induced vasodilation. Although this model supplies us with a basic understanding, it is in no way complete. Of these projects that have contributed to this model, one of the most interesting lines of investigation into cGMP metabolism has been that of its regulation. Nearly all intracellular messengers are subject to some form of regulation and cGMP is no exception. As suggested above, two points for regulation of cGMP are its synthesis by GC, and its degradation by PDE. The regulation of GC and PDE activity in the cascade of events leading to vasorelaxation is an important factor for the overall vascular response of an organism. Considering the importance of the cGMP synthesis/degradation relationship, a better understanding of the enzymes responsible for each is in order.

Guanylate Cyclase

Guanylate Cyclase is the intracellular enzyme responsible for the synthesis of cGMP from GTP. GC is found in two biochemically different forms. One form of GC is membrane bound (particulate) and the other is cytoplasmic (soluble). The particulate form of GC appears to be evolutionarily a very old

5

enzyme which can be found in most animal species and monocellular systems [Tremblay, et al. 1988]. The membrane bound form of GC must be activated directly by stimuli [Goy, 1991]. The soluble form of GC has been found in most mammalian cells. It is this form of GC which is responsible for the production of cGMP in the vascular smooth muscle. Unlike the particulate GC, soluble GC contains an associated heme group, and must be activated indirectly via EDRF (NO), or a number of other compounds (peroxides, unsaturated fatty acids such as arachidonate, or certain lipids such as lysophosphatidyl choline and oleic acid) [Goy, 1991] [Pearce and Harder, 1994]. It is thought that NO activates soluble GC by interaction with its heme component [Gerzer, et al., 1982]. Although it is conceivable that other agents capable of interacting with heme (other free-radicals) could produce changes in GC activity, in 1987 Ignarro proposed that NO was physiologically the most important and abundant of these agents [Ignarro, et al., 1987]. Since that time a large body of work has been published which supports this view.

Although both forms of GC are highly characterized, dramatic evidence of direct regulation has never been shown for the soluble form. The existence of some cGMP regulation via GC activity has been proposed for NO and other free-radicals. Any alteration in these diffusible substances would increase the response of GC. This is based on the fact that GC is sensitive to those factors which interact with the iron-containing heme group in soluble GC. Related to this form of regulation would be the the formation of free-radical scavengers such as superoxide anion and/or any oxidizing compound in the tissue. Formation of these scavengers would effectively inhibit the same GC activity [Lincoln, 1989].

Cyclic nucleotide PDEs are the intracellular enzymes responsible for the degradation of both cAMP and cGMP. Although nearly all cGMP within the cell undergoes hydrolysis by PDE, intracellular levels of cGMP may also be reduced by the release of cGMP into extracellular space, this however accounts for very little of the total cGMP removed from the cell [Schini et. al., 1989]. At present there are over 20 known isozymes of cyclic nucleotide specific PDEs found in various types of tissue, fibroblasts, and platelets [Beavo and Reifsnyder 1990]. These isozymes have been divided into five distinct families based on their biochemical characteristics (type I through V.) **[Table 1]**. Not only are the biochemical characteristics of these isozymes

PDE Type	Substrate	Inhibitor
I	cAMP & cGMP Ca2+ / Calmodulin-dependent	Vinpocetine EGTA
II	cAMP & cGMP cGMP-stimulated	No specific
III	cAMP & cGMP cGMP-inhibited	Indollidan Cilostamide Quazinone
IV	cAMP cAMP-specific	Rolipram
V	cGMP cGMP-specific	Zaprinast (M&B22948)
All	nonselective	IBMX Theophyline

Table 1: Phosphodiesterase Isozyme Families and Some Selective Inhibitors. This table is not meant to be complete. Most agents noted show at least a 20-fold selectivity for the family listed. Information presented in this table has been extracted from Beavo and Reifsnyder, 1990.

different, but comparison of the gene sequences for each has confirmed the presence of multiple genes that code for the different family members [Swinnen, et al., 1989] [Le Trong, et al., 1990]. It should be noted that no standardized nomenclature existed for the PDE types until 1990 [Beavo and Reifsnyder 1990]. Before this time each PDE isozyme was referred to by the order in which they were eluted from a DEAE column. To complicate matters even further, different extraction techniques yielded different PDE isozymes. As a result much of the early literature is difficult to decipher in regards to the PDE type being described. Despite this, a number of researchers have shown these PDE isozymes and families to be very tissue specific [Weishaar, et al., 1986][Silver, et al., 1988][Souness, et al., 1990]. This heterogeneity in PDE distribution, combined with the existence of drugs designed to inhibit specific PDE types, has generated considerable interest for clinical use [Polson, 1990]. In a traditional clinical setting, high blood pressure is treated by the use of beta-adrenergic blockers such as *propranolol* and *atenolol*. Unfortunately, vasodilatation induced in such a manner is systemic. While this has the desired effect of lowering blood pressure, it also effects systems which require higher blood pressures for optimum performance, such as the kidneys and lungs. The pharmacological development of a PDE inhibitor targeted at specific tissue would be a valuable clinical tool. This possible therapeutic application has fueled a new interest in PDE activity and its regulation.

The complexity of the PDE families have also produced major problems in studying these enzymes and their regulation. Despite the difficulties involved, a major precedent for dramatic hormonal regulation of cGMP PDEs has been shown in the retina [Stryer, 1986 &1991]. In retinal rods and cones,

the capture of a photon of light increases the rate of cGMP breakdown, and the stimulus is thus transduced into decreased intracellular cGMP levels. Additional examples of complex hormonal regulation of cyclic nucleotide PDEs have also been shown by Dumas, et al., (1988) and Laugier, et al., (1988) in the quail oviduct. The knowledge that PDEs can be, and are regulated in a complex fashion is an indication of the important role these enzymes may play in many cellular processes. Short-term regulation and long-term cellular modulation by different stimuli and maturation might be one function of PDEs [Conti, et al., 1991].

Although a great deal of research has been conducted into cGMP in vascular smooth muscle over the last 15 years, relatively little direct work has been done to investigate cGMP metabolism, and even less concerning the effect of maturation on cGMP metabolism. During maturation, a vast number of different hormones, steroids, proteins and other biochemical compounds are produced. Such a wide range of change would encompass an even larger sphere of affected systems. Not surprisingly, maturational changes have been observed in the endothelium of vascular smooth muscle [Vane, et al., 1990] [Pearce and Harder, 1994]. These changes affect the smooth muscle and its response to relaxation stimulation. It would therefore stand to reason, that the cGMP metabolism in vascular smooth muscle could be directly modulated by maturation as well. Dramatic evidence which supports this hypotheses has been shown by Pearce, et al., [1994] in the sheep common carotid artery **[Fig. 2]** (data reproduced by permission of W. J. Pearce). Utilizing isolated artery segments from newborn and adult animals, Pearce examined the effects of maturation on the dose-response and dynamic relationships between

relaxation and cGMP synthesis. The amount of relaxation produced by a cumulative dose response to the cGMP dependent vasodilator SNAP, is nearly 40% greater in the newborn than the adult. This evidence suggests a
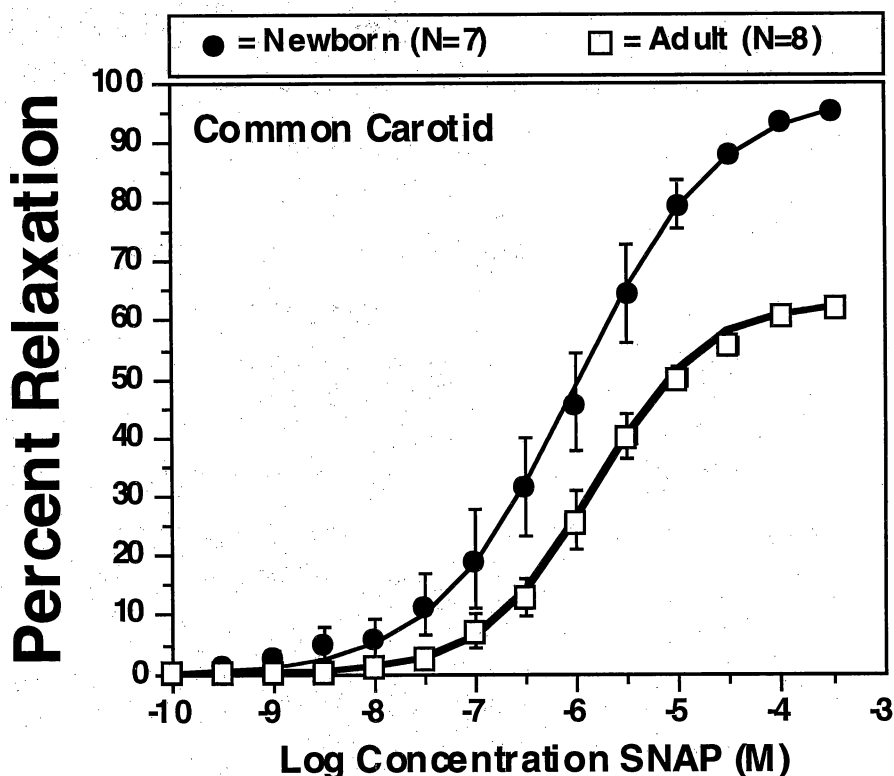
Figure 2: <u>Cumulative Dose Response to SNAP in the Common Carotid Artery for Both Newborns and Adults</u>. Arteries were precontracted with 10 μM serotonin and 20 μM histamine. The average relaxant responses were calculated as the precent relaxation of initial contractile tone, and are given witn standard errors. N = 7 in the newborn, and 8 in the adult. *Data reproduced by permission of W. J. Pearce.*

larger pulse of cGMP, which in turn would lead to a greater amount of phosphorylation by G-kinase, which could ultimately convey a greater propensity for relaxation in the newborn. This marked change in relaxation response may result in a significant physiological detriment for the newborn.

Newborns suffer a high incidence of cerebrovascular complications

postpartum [Pape, 1989] [DelToro, et al., 1991]. This enhanced ability for

vasorelaxation in the newborn may result in such complications as asphyxia

and transient hypertension [Pearce, et al., 1991 & 1994]. Due to problems

such as these, and the newly founded interest in the clinical use of PDE

inhibitors, a legitimate need has arisen for investigation into cGMP metabolism

alone, and as a function of age.

Project Introduction

This project has been designed with two specific aims in mind. The first,

to examine and compare GC and PDE activity in newborn and adult sheep

common carotid arteries. The second, to determine if compartmentalization

affects PDE activity.

In the first section, I have addressed a question which was raised in a

previous study by Pearce et. al., (1994). They observed a significant difference

in the basal cGMP levels of the common carotid artery between newborn and

adult sheep. Basal cGMP levels were elevated in the newborn as compared to

the adult. This difference in the basal levels may somewhat account for the

increased efficacy of relaxation seen in Fig. 2. Furthermore, this observation

may represent a significant difference in the overall cGMP metabolism which is

modulated by maturation. Given the central importance of cGMP metabolism

and its potential for modulation by maturation, the first section of this study

addresses the hypothesis that maturation modulates cGMP metabolism by

investigating the possible mechanisms involved in this phenomenon. I have

hypothesized four likely mechanisms to explain Pearce's observation; 1) Basal

EDRF is greater in the newborn, compared to the adult, 2) The synthetic capacity of GC is greater in the newborn, compared to the adult, 3) PDE activity is lower in the newborn, compared to the adult, or 4) The difference between synthesis/ degradation is similar in both newborn and adult, and it is the absolute levels of cGMP which differ.

Evidence which shows that compartmentalization plays a role in GC activity has been thoroughly summarized by Waldman and Murad [1987], but similar information regarding PDE activity has not been forthcoming. To address my second aim, I have conducted this study in both homogenized and isolated intact common carotid arteries from both age groups. This experimental design, allows me to address the recent concerns raised in the literature that the traditional broken cell techniques used for studying PDE activity, may actually produce physiologically irrelevant information [Schoeffter, P. et al., 1989][Ahn, H. O., et al., 1989][Barber, R., et al., 1992]. The basic techniques I used for the broken cell preparations (crude homogenates) are well established [Wells, et al.1974]. Unfortunately, no experimental design has been reported which allows for the measurement of PDE activity in intact tissue. In order to study PDE activity in an intact preparation, I developed a novel technique to estimate both GC and PDE activity simultaneously in a single intact section of artery.

METHODS

Experimental Procedures

I obtained common carotid arteries (COM) from young non-pregnant adult sheep (age 18-24 months) and newborn lambs (age 3-5 days). Tissue from adult animals was obtained from a local slaughterhouse within 4 hours of slaughter, and kept packed in ice until dissection. Tissue from newborn animals was obtained from lambs brought into the facility, and sacrificed with a lethal injection of sodium pentobarbital on the day of the experiments.

The following is a detailed explanation for the initial treatment of the arteries used in this study. This procedure was originally developed by Pearce et al.,1991. three or four cm sections of arteries were cleaned of all connective tissue and adipose tissue. Except where noted, each segment was mechanically denuded of its endothelium using a roughened, large gauge blunt-end hypodermic needle, and then flushed with water. By removing the endothelium prior to the experimental procedure, I was able to eliminate any unwanted effect of EDRF which would result in an uncontrolled stimulation of the vascular smooth muscle. The vessel segments were cut into 3 mm ring segments and each vascular ring was mounted on paired wires between a force transducer (Kulite BG-10) and a post attached to a micrometer (used to vary resting tension). During all experiments, data was continuously digitized, normalized, and recorded using an on-line computer **[Fig. 3]**.

In a Krebs-bicarbonate solution containing; 122mM NaCl, 25.6mM $NaHCO_3$, 5.56mM dextrose, 5.17mM KCl, 2.49mM $MgSO_4$, 1.60mM $CaCl_2$, and 0.027mM disodium EDTA, continuously bubbled with 95% O_2, 5% CO_2

and maintained at 38.5°C (normal ovine core temperature), the freshly mounted arteries were slowly and repeatedly stretched until optimum baseline tensions of 1g [Pearce et al., 1991] remained stable for at least 30 min. In order to replenish the vessel's intracellular stores of Ca^{2+}, the arteries were then contracted with an isotonic potassium Krebs solution containing 122 mM K^+ and 31 mM Na^+. After peak tensions were reached, the arteries were washed with normal sodium Krebs and allowed to re-equilibrate to baseline tension for another 30 min.
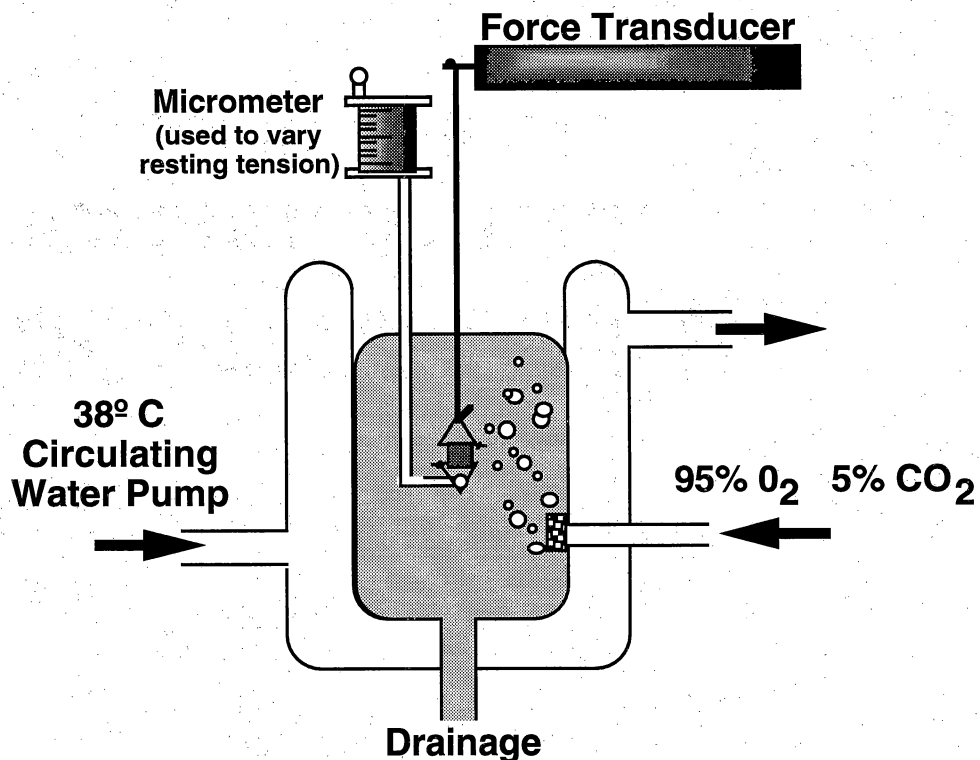

Figure 3: The Isolated Vessel Bath. Vessel segments were cut into 3 mm ring segments and each vascular ring was mounted on paired wires between a force transducer (Kulite BG-10) and a post attached to a micrometer (used to vary resting tension). During all experiments, data was continuously digitized, normalized, and recorded using an on-line computer.

cGMP Time Course

In order to determine if the difference in newborn and adult basal cGMP levels observed by Pearce et al., 1994, were due to a difference in basal EDRF levels, vessels which were both endothelium intact and endothelium denuded, were flash frozen in liquid nitrogen at baseline condtions after a K^+ contraction. cGMP samples were taken at this point based on the previous validation work by Pearce et al., 1994, which has shown that basal cGMP levels remain unchanged before or after a serotonin or K^+ contraction.

To measure the normal cGMP time course, six segments from each animal studied were used. Once a stable contraction had been achieved using 1μM serotonin (5-HT), each vessel was treated with 10 μM (EDmax) aqueous S-nitroso-N-acetyl penicillamine (SNAP, an exogenous source of nitric oxide) and flash frozen at t = 0s, 20s, 40s, 60s, 80s, or 100s. In order to estimate the GC activity alone, these experiments were also repeated with a 4 min pre-incubation in 300 μM of 3-isobutyl-1-methylxanthine (IBMX, a total inhibitor of phosphodiesterase) dissolved in DMSO, prior to the treatment with 5-HT and SNAP. The frozen vessels were subsequently homogenized and assayed for cGMP as described below.

Crude Homogenate Preparation

In light of concerns raised in the literature that traditional broken cell techniques for studying PDE activity may produce physiologically irrelevant information, I performed a set of experiments to compare intact and broken cell

determinations of PDE activity. For the broken cell technique I chose a slightly unorthodox technique. Rather than measuring the appearance of product in my preparation (in this case GMP), I chose to measure the disappearance of substrate (cGMP). The reason I chose this approach, was based on two factors. 1) The assay developed by Wells, et al., [1975] to measure the appearance of GMP, is extremely complicated, time consuming, and expensive. This assay also requires equipment which I do not have at my disposal. 2) The sensitivity of my already existing cGMP assay was extremely high. This assay can detect down to 1.5 fmol cGMP in a 75µl sample. This range of sensitivity gave me confidence that I could accurately measure minute changes in substrate concentration.

Approximately 20 - 30 mg (wet weight) sections of COM were homogenized in a 40 mM Tris-HCl (pH 7.5), 1.6 mM $CaCl_2$, 2 mM $MgCl_2$, 1 mM dithiothreitol buffer at 4ºC [Wells, et al., 1975] [Weishaar, et al., 1986]. Also added to the homogenization buffer was a mixture of protease inhibitors; 76.8 nM aprotinin, 83 mM benzamidine, 1 mM iodoacitamide, 1.1 µM leupeptin, 7 µM pepstatin A, 0.23 mM phenylmethanesulfonyl fluoride (PMSF) [Campbell, et al., 1984] for a total of 2 ml. Samples were centrifuged at 3000g for 30 min. An aliquot of the resulting supernate was assayed for Tris-HCl soluble proteins as described below. The remaining supernate was divided into six, 250µl samples and placed into tubes kept at 4ºC. In order to remove any endogenous cGMP from the preparation, each tube was individually warmed in a 37ºC water bath for a minimum 30 min "clearing period", before the addition of substrate (10 µmol cGMP in a 0.05M sodium acetate buffer (pH5.8) containing sodium azide [Amersham Corp., Illinois]). Early attempts at

this protocol without the clearing period, produced varying starting levels of cGMP. The absolute length of the clearing period did not seem to be relevant, so long as it exceeded a minimum of 30 min. The concentration of the substrate itself, was approximately 10x greater than reported values of *Km* for PDE. With the addition of the substrate, each tube was allowed to incubate for t = 0s, 5m, 10m, 15m, 20m, or 25m. At the end of the incubation period 2 ml of 6% TCA was added to each tube. At this point the samples were centrifuged, ether washed, lyophilized and assayed for remaining cGMP.

cGMP Synthesis and Degradation in Intact Segments

In order to preserve intracellular compartmentalization, thus more closely emulating the *in vivo* system of the intact artery, I have developed a unique method for deriving both PDE and GC activity simultaneously in a minimal amount of tissue from a single animal. I refer to this technique throughout the study as the *5-30 protocol*.

From each animal studied, two sets of vessels, each consisting of three adjacent vessel segments were used. To determine the synthetic capacity for cGMP (SYN), the first set of vessels were pre-incubated in 300 µM IBMX for 4 min before the administration of SNAP and then flash frozen. The first segment was frozen at t = 5s, the next at t = 15s, and the last at t = 30s. To determine the net total of cGMP synthesis and degradation combined (NET), the second set of vessels were treated only with 10 µM SNAP, and then similarly frozen. The frozen vessels were subsequently homogenized and assayed for cGMP as described below. The slope of the change in cGMP accumulation (pmol/mg

17

protein/min) between 5s, 15s, and 30s, was used to calculate the rates of SYN and NET. Degradation (DEG) was calculated as the algebraic difference between SYN and NET. Because this technique calculates PDE activity as the algebraic difference between SYN and NET, rather than directly measuring PDE activity, the PDE activity determined in this manner is expressed as an estimation of activity.

One of the most important aspects of this protocol is time points at which I chose to take my measurements at. The time points that I ultimately chose were based upon the following assumption; Before peak cGMP is reached, synthesis must be greater than degradation, as peak cGMP is reached, synthesis must equal degradation, and finally as the cGMP level returns toward basal values, synthesis is less than degradation. Keeping this in mind, I chose the time points for this protocol so that both the synthetic and degradative components were active at the same time. In addition to this consideration, I chose not to take my first measurement at t = 0s, rather I took it at t = 5s. This was in order to allow the exogenous NO released from SNAP, time to defuse into the artery. This would ensure that all measurements would be in enzymatically active arteries.

Cyclic Nucleotide and Protein Determinations

Frozen artery segments were stored at -80ºC until assay, at which time they were individually homogenized in 1 ml ice-cold 6% trichloroacetic acid using a motor driven ground glass pestle and mortar (Lurex, Vineland, New Jersey). After centrifuging the homogenates for 60 min. at 3000g, the resulting

18

pellet was used for protein determination and the decanted supernates for subsequent cGMP assay.

Determination of protein content was identical to that previously described by Pearce et al.,1991, except for samples from the crude homogenate preparation (see below). For each protein sample, the pellet was resuspended in 1.0 M NaOH at 37°C for 60 min. This method of extraction is designed to exclude connective tissue and structural proteins, as previously shown by others [Furuto et al., 1987] [Soskel et al., 1987]. After resuspension, the samples were centrifuged again for 60 min. at 3000 g. Aliquots of the resulting supernates were then neutralized with an equal volume of 0.9 M HCl. In order to avoid protein aggregation, the samples were further diluted with 125 mM urea at a 5:1 ratio of urea to sample. The protein was then quantified using the BioRad's coomassie brilliant blue protein dye (cat#500-0006). Bovine serum albumin served as the reference for the standard curves determined with each set of unknowns. As has been previously shown, this assay produces protein values which are both consistent and uniform in the vessel type studied [Pearce et al., 1991].

The aliquots of Tris-HCl soluble proteins from the crude homogenate preparation did not undergo NaOH resuspension or neutralization. These samples were diluted directly with 125 mM urea at the same 5:1 ratio before the addition of the Bradford dye.

To prepare the corresponding original supernates for cGMP determination, each sample was washed with water saturated diethyl ether a minimum of three times to remove the TCA used to originally homogenize the samples. Any remaining ether was allowed to evaporate, and aliquots of the

19

aqueous phase were then lyophilized and stored at 4ºC until assayed.

cGMP content for each sample was determined using commercially available radio-immune assay (RIA) kits [RPA 525 Amersham Corp., Illinois]. The following is a brief summary of the Amersham cGMP RIA assay. The assay is based on the competition between unlabeled cGMP and a fixed quantity of ^{125}I-labeled cGMP for a limited number of binding sites on a cGMP-specific antibody. With a fixed amount of antibody and radioactive ligand, the amount of radioactive ligand bound by the antibody will be inversely proportional to the concentration of added non-radioactive ligand. The antibody bound cGMP is then reacted with the Amerlex-M™ second antibody reagent which contains a second antibody that is bound to magnetizable polymer particles. The antibody bound fraction is separated by centrifugation. When the unbound radioactive supernate is decanted off from the pellet, measurement of the radioactivity in the pellet enables the amount of labelled cGMP in the bound fraction to be calculated. The concentration of unlabeled cGMP in each sample is then determined by interpolation from a standard curve.

Normalization

All reported values for cGMP were ultimately normalized to give µmol cGMP / L cell water. Total water content for each age group was determined separately, as the algebraic difference between the tissue's wet weight and its dry weight. Subsequent protein assays were performed on the dry tissue using ether the NaOH or Tris-HCl extraction techniques to determine the ratio of protein to total tissue water. The ratio of cellular protein to cell water was

determined using the formula:

$$\%P\ /\ \%W_i\ *\ (100\text{-}\%W_t)\ /\ \%W_t$$

where %P = %dry weight protein, $\%W_i$ = %intracellular water, and $\%W_t$ = total %wet weight water. Both %P and $\%W_t$ were measured directly. The value used for $\%W_i$ was taken from published values [Cox, et al. 1976]. These ratios for both NaOH soluble protein and Tris-soluble protein were used to convert all cGMP values into units of μmol cGMP per liter of cell water. By normalizing to cell water content, rather than raw protein, I was able to express cGMP as a concentration, thus avoiding maturational changes in protein encountered when comparing the newborn to the adult.

RESULTS

All reported values of n refer to the number of animals, and not the number of segments. All values are reported as means ± SE. Unless noted, all values for cGMP are expressed as µmol cGMP · L cell water $^{-1}$. Statistical significance was determined with a student's T test, where $p < 0.01$ equals a significant difference.

Basal EDRF

As indicated in **Fig. 4**, the basal level in the adult COM with intact
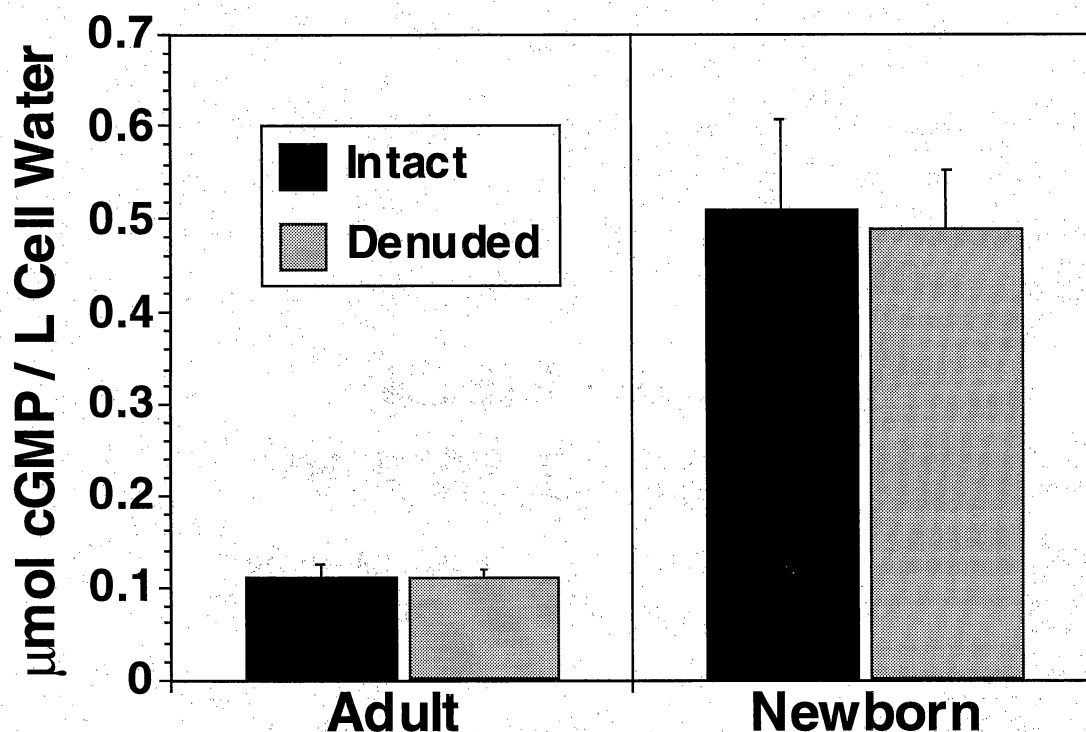

Figure 4: Basal cGMP in Newborns and Adults ± Endothelium. All values are given as means ± SEM. N = 12 for adults and 10 for newborns in endothelium intact segments. N = 15 for adults and 8 for newborns in endothelium denuded segments.

endothelium shows no significant difference (0.11 ±0.017) from the paired endothelium denuded adult COM. (0.11 ±0.007). Consistent with this finding, the corresponding newborn values for the COM with intact endothelium (0.508 ±0.098) and its paired endothelium denuded COM (0.488 ±0.064) also showed no significant difference. For both intact and denuded, the newborn basal cGMP levels were significantly higher than the adults.

IBMX Validation

Before I could utilize the PDE inhibitor IBMX, my first step was to determine the concentration of IBMX needed to completely inhibit PDE activity in my preparations. Previously reported effective doses for IBMX ranged from 1 µM to 1 mM. In order to chose the proper dosage, I took measurements of cGMP in the presence of varying concentrations of IBMX (3 µM, 30 µM, and 300 µM,) over a 12 min time period in endothelium intact segments. I found that all three concentrations produced similar stable plateau values of cGMP (data not shown). Because the concentrations tested showed a similar capacity for cGMP inhibition, I chose the average dosage reported in the literature, 300 µM IBMX, as my experimental concentration **[Fig. 5]**.

Crude Homogenate Preparation.

Using the crude homogenate approach, I first examined the rates of cGMP degradation by total PDE with, and without IBMX for both age groups. The rate of degradation was calculated for each animal in each age group, as the slope of the disappearance of the cGMP substrate over the first 15 minutes of incubation. Individual animals were included or excluded from the analysis using values of r^2 criteria for fit. Any animal expressing an r^2 value of <0.950 (95% confidence level) was excluded. Finally, the individual slopes for each
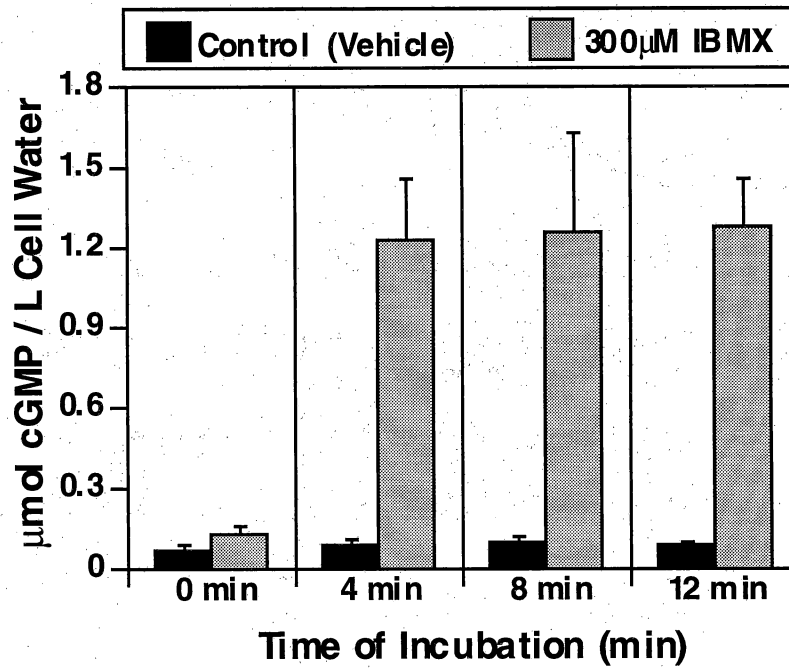
Figure 5: <u>Validation of the Effect of 3-isobutyl-1-methyl-xanthine (IBMX) in Adult Endothelium-Intact Common Carotid Arteries</u>. Vehicle for IBMX is DMSO. All values are given as mean ±SEM. N = 4 for IBMX. N = 7 for control.

age group were averaged and plotted using the slope to back-calculate the mean ± SE for each time point (0m, 5m, 10m, and 15m). This technique revealed a significantly greater degradative capacity in the adult COM (2.406 ±0.224 µmol cGMP · L cell water $^{-1}$ · min $^{-1}$) than the newborn (1.407 ±0.157 µmol cGMP · L cell water $^{-1}$ · min $^{-1}$) $(p <0.01)$ [**Fig. 6**]. Although measurements of cGMP were also taken at 20 and 25 minutes, these time points were not included in the analysis. These time points at the extreme end of the incubation displayed a non-linear nature. This would tend to indicate that the concentration of the cGMP substrate had fallen below the optimum PDE Km.
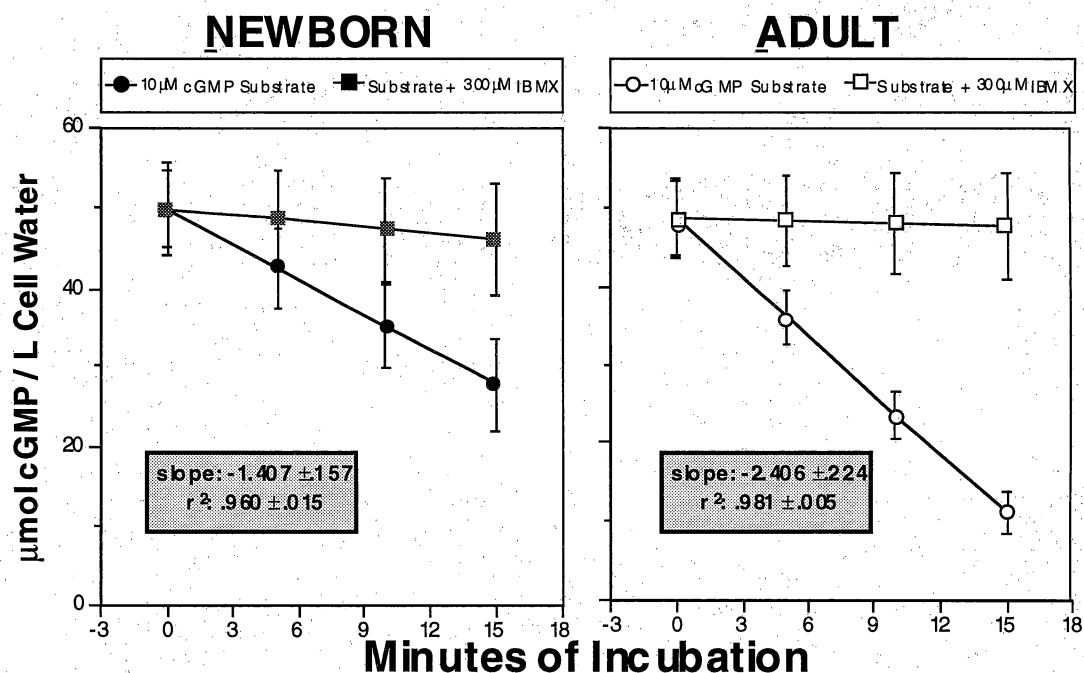

Figure 6: Rates of cGMP Degradation in Crude Homogenates. All values are given as mean ±SEM. N = 5 for IBMX + cGMP, and 9 for cGMP in the newborn. N = 5 for IBMX + cGMP, and II for cGMP in the adult.

cGMP Time Course:

When intact artery segments from each age group were treated with a single dose of 10μM SNAP, cGMP levels increased rapidly and peaked between at 60s (1.41 ±0.191; adult COM) and 80s (1.6 ±0.308; newborn COM). After peak levels were reached, cGMP returned towards baseline levels by 100s **[Fig. 7]**. Although no significant differences were observed in
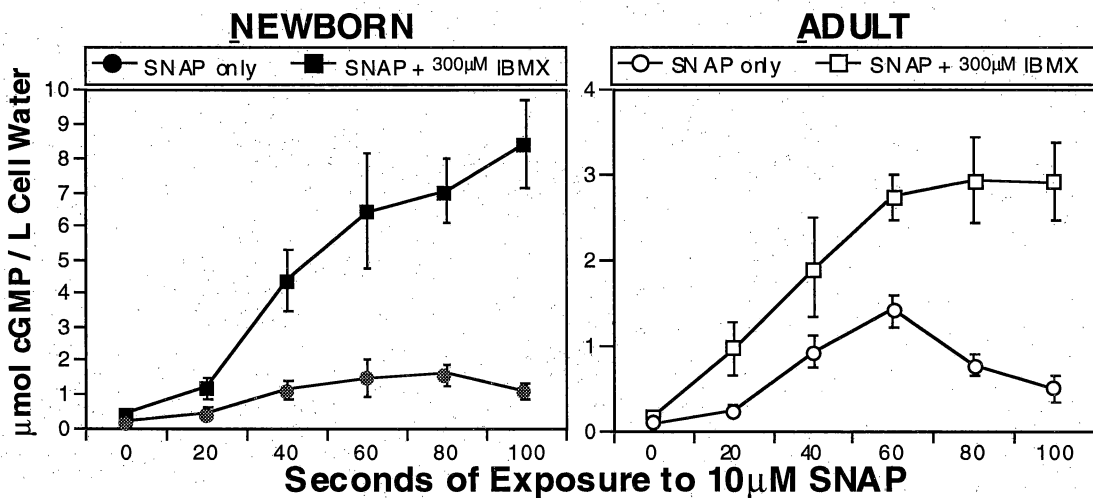
Figure 7: <u>Normal SNAP-Induced cGMP Time Course, Compared With the Effects of IBMX on SNAP-Induced cGMP Accumulation</u>. All values shown are given as mean ± SEM. The total cGMP synthesized in the newborn was143.39 μmol cGMP · L cell water $^{-1}$ and 85.54 μmol cGMP · L cell water $^{-1}$ in the adult. Total synthesized cGMP was determined by integrating the area under the normal time course curve. N = 14 in the newborn and 10 in the adult for SNAP. N = 9 in the newborn and 8 in the adult for SNAP/IBMX.

cGMP peak levels between age groups, a significant difference was observed in the total cGMP synthesized in each age group. The total cGMP synthesized in the newborn (143.39) was significantly higher than in the adult (85.54). Total synthesized cGMP was determined as the integrated area under the time course curve.

When intact artery segments from each age group were pre-treated with 300µM IBMX before the addition of 10µM SNAP, cGMP levels increased rapidly and reached plateau values within 100s. Once reached, this plateau was stable for at least 12 min (as was shown in Fig 5.). Adult arteries reached a stable plateau of approximately 2.917 ±0.502 by 100s. The cGMP levels in the newborn reached a plateau of approximately 8.416 ±1.338 by 100s. Although both age groups achieved a plateau, the level of cGMP accumulation was significantly greater in the newborn than the adult.

PDE and GC estimation in whole artery segments:

To preserve compartmentalization, I developed a technique which enables me to estimate the rates of cGMP degradation in whole, unbroken arteries. This technique also enables me to make a simultaneous estimation of the rate of cGMP synthesis from the same animal **[Fig. 8]**. Over the first 30s of exposure to SNAP, both the newborn and adult have a similar net rate (NET) of cGMP synthesis - degradation (0.056 ±0.007 and 0.051 ±0.01 µmol cGMP · L cell water $^{-1}$ · sec $^{-1}$, respectively). Over the same exposure period, the newborn displays a higher synthetic rate (SYN) than the adult (0.127 ±0.019 and 0.099 ±0.021 µmol cGMP · L cell water $^{-1}$ · sec $^{-1}$, respectively) When the rates of degradation (PDE activity) for each age group are calculated as the algebraic difference between SYN and NET, the newborns display a higher rate of degradation when compared to the adults (-0.071 ±0.014 and -0.048 ±0.013 µmol cGMP · L cell water $^{-1}$ · sec $^{-1}$, respectively). The technique used for determining the individual slopes was identical to that used for the crude homogenate preparation.
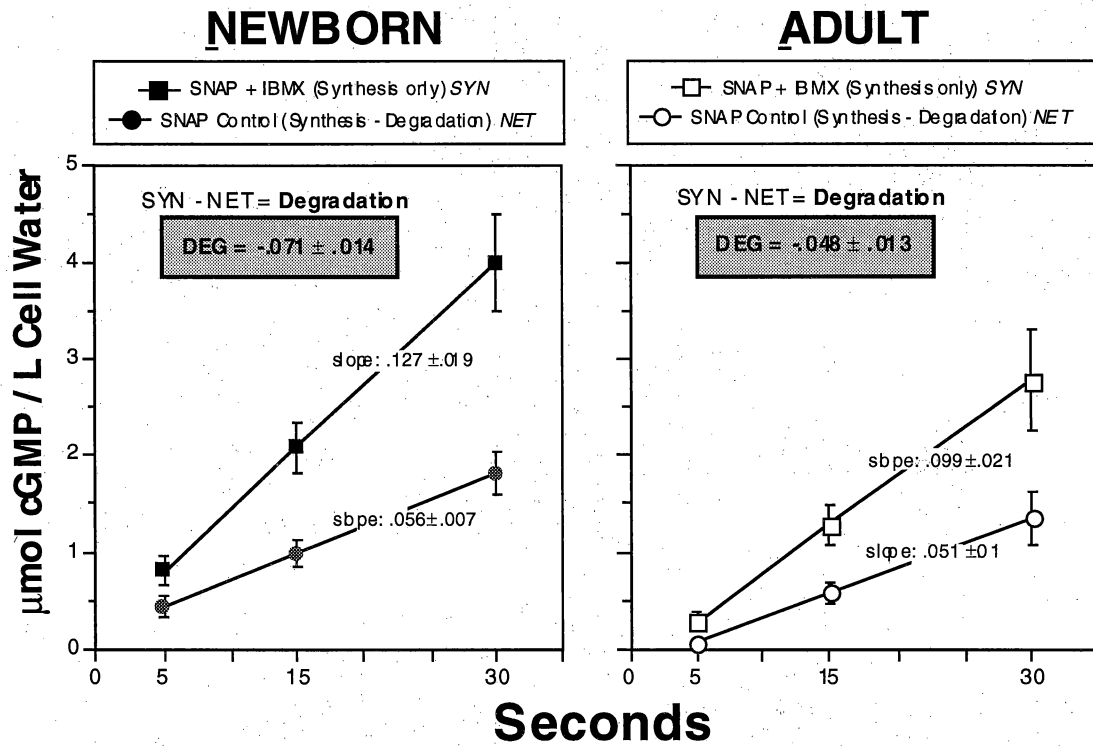
Figure 8: <u>Rates of cGMP Accumulation and Degradation in Intact Arteries</u>. All values are given as mean ±SEM. N = 8 in the newborn and 13 in the adult.

DISCUSSION

Part I: cGMP Metabolism

In the introduction, I have suggested several likely explanations for the age-related differences in basal cGMP levels. Those explanations are; 1) *Basal EDRF is greater in the newborn, compared to the adult,* 2) *The synthetic capacity of GC is greater in the newborn, compared to the adult,* 3) *PDE activity is lower in the newborn, compared to the adult,* or 4) *The difference between synthesis / degradation is similar in both newborn and adult, and it is the absolute levels of cGMP which differ.* When considering the results of this project, I believe that I can eliminate the first of these explanations. Based on **Fig. 4** in which I have removed the endothelium from the ring segments and compared the basal cGMP levels with endothelium intact ring segments, I have clearly demonstrated that the endothelium does not effect the basal cGMP levels.

In order to address the second and third explanations, I had to conduct experiments which could separate each of these elements. Traditionally this would be accomplished in a broken cell preparation, in which the enzyme in question would be isolated, purified, and then tested in the presence of its substrate for product appearance. Unfortunately this is not a practical approach in the case of soluble GC. Soluble GC activity is somewhat affected by compartmentalization [Waldman and Murad,1987]. So in order to address the second explanation: 2) *The synthetic capacity of GC is greater in the newborn, compared to the adult,* I chose a pharmacological approach in intact tissue. By inhibiting the degradative activity of PDE with IBMX, I was able to

measure the total accumulation of cGMP over the first 100s after exposure to SNAP **[Fig. 7]**. I used this cGMP accumulation as a direct indication of GC activity. The level of cGMP accumulation was much greater in the newborn than the adult. This may indicate a greatly enhanced GC capacity in the newborn relative to the adult. If this is true, this data would tend to support the second explanation. However, a certain question casts some doubt upon this as the sole explanation. Is this truly a measurement of CG activity, or is it an indication that newborn and adult GTP levels are different? Substrate exhaustion of GTP by GC could easily explain the observed differences in plateau cGMP values. Because newborns are metabolically more active than adults, it is conceivable that greater levels of GTP do exist in the newborn compared to the adult. An extensive search of the literature yielded no information on this possibility. As such, the second explanation can neither be proven nor eliminated at this point. In light of this consideration, what can be stated is; whether due to a difference in GC or substrate availability, the newborn does have a higher capacity for cGMP synthesis compared to the adult in an *in vivo* situation.

When I began to consider the third explanation: *3) PDE activity is lower in the newborn, compared to the adult,* I encountered the problem of PDE compartmentalization. The likelihood that PDE activity was affected by compartmentalization was extremely high. Despite this likelihood, studies involving PDE families and subtypes have all previously been conducted in a traditional broken cell preparation. To complicate matters even further, the published values for PDE *Vmax* and *Km* are both wide and varied. This variation could be accounted for by the extraction and isolation techniques

utilized by each study. Rather than simply repeating similar experiments to investigate possible maturational changes in PDE activity, I chose to investigate PDE activity in both intact and homogenized arteries. For the broken cell technique I chose to use crude homogenates rather than an isolated and purified enzyme preparation for three reasons. 1) A crude homogenate preserves any possible enzyme co-factors that may be required by PDE for optimum activity. 2) Enzyme purification requires large quantities of tissue, which necessitate the pooling of tissue from several animals. This technique makes the determination of PDE activity from a single animal impossible. 3) In an *in vivo* system, the total amount and velocity of cGMP degradation, is a function of the combined activities of all PDE types present. An isolated PDE preparation only yields data for a single PDE species, and does not accurately reflect the *in vivo* system.

Utilizing this crude homogenate approach, I was able to examine the rates of cGMP degradation by PDE with and without IBMX for both age groups. This technique revealed nearly a two fold greater degradative capacity in the adult **[Fig. 6]**. Based solely on this data, it would appear that the lower basal levels found in the adult are due to the adult's greater PDE activity. Data collected in this manner would tend to support the third explanation Although I was able to determine the apparent rates of degradation for each age group with this technique, these rates are only an expression of the *Vmax* for PDE. Whereas I could control the starting concentrations of substrate in my preparations, the same is not true for the starting concentrations of PDE. *Vmax* values are extremely sensitive to enzyme concentrations and characteristics. Both of these factors can change from animal to animal, and from preparation to preparation.

I also chose to investigate PDE activity in intact arteries. The technique that I developed to accomplish this is the is the *5-30 protocol*. The advantage of this technique over the crude homogenate approach, is that not only does it enable me to estimate the rates of cGMP degradation in whole, unbroken arteries, it also enables me to make a simultaneous estimation of the rate of cGMP synthesis from the same animal. Although the *5-30 protocol* is an *in vitro* approach, it more accurately duplicates the *in vivo* situation and eliminates much of the animal to animal variation encountered in the homogenate approach. If I apply the *5-30 protocol* to examine the degradative component alone, the estimated PDE activity is higher in the newborn compared to the adult **[Fig. 8]**. When this data is compared with that from the homogenate experiments, the results produced are completely opposite. In order to decide which of the two preparations best represent the *in vivo* situation, I have gone back to the results of the second question. If the results from the second question can be accepted as accurately reflecting the *in vivo* situation due of the intact nature of the experiment, I must accept the intact PDE preparation as the preparation which most accurately reflects the *in vivo* situation. In doing so, the results of the intact PDE preparation must therefore eliminate the third explanation for the age-related differences in basal cGMP levels. Although accepting the intact preparation has eliminated an explanation, it has also provided a clue that supports the fourth explanation.

I believe that my final explanation: *4) The difference between synthesis / degradation is similar in both newborn and adult, and it is the absolute levels of cGMP which differ,* represents the best mechanism for the age-related differences in basal cGMP. In order to justify this explanation, I must utilize

data from both the *5-30 protocol* [**Fig. 8**], and the normal cGMP time course [**Fig. 7**]. Now when I consider *5-30 protocol* [**Fig. 8**], I have included all of the components in the technique. Both synthesis and degradation are higher in the newborn compared to the adult, but the net total of cGMP synthesis and degradation combined is nearly the same. This indicates that regardless of the absolute rates of the synthesis or degradation, when fully activated, the net effect of the enzymes are the same.

Further support for this explanation can be found in the normal cGMP time course. During the dynamic measurements of cGMP responses to SNAP seen in **Fig.** 7, the age-related differences observed under basal conditions do not persist at peak cGMP levels. Because the peak values in the newborn are similar to those of the adult, the difference between synthesis and degradation during maximal activation appears unaffected by age. It remains possible, however, that the absolute levels of both synthesis and degradation are quite different in the newborn and adult. This is indicated by the significant difference observed in the total mass of cGMP synthesized in each age group. The total cGMP synthesized in the newborn was higher than in the adult. Because the difference between synthesis/ degradation is similar in both newborn and adult, the inital rise, time to peak, and cGMP level at peak are all similar. Furthermore, the relationship between synthesis and degradation of cGMP is similar in both the adult and newborn arteries, however, the total mass of cGMP synthesized following a given stimulus is greater in the newborn than adult arteries, and hence the impact of the same amount of degradation is correspondingly less. This results in a net greater mass of cGMP synthesis, greater secondary phosphorylation, greater attenuation of vascular tone and

ultimately more prolonged relaxation state in the newborn as compared to the adult arteries, for the same stimulus. The higher basal levels of cGMP seen in the newborn arteries reflects the lesser impact of degradation of cGMP in the newborn as compared to the adult vessels. This observation combined with the findings of the *5-30 protocol* lends considerable support to the fourth explanation. A better graphic representation of this statement can be seen in **Fig. 9**. which summarizes **Fig. 8** and helps to more clearly illustrate the difference in the absolute levels of cGMP between the age groups.
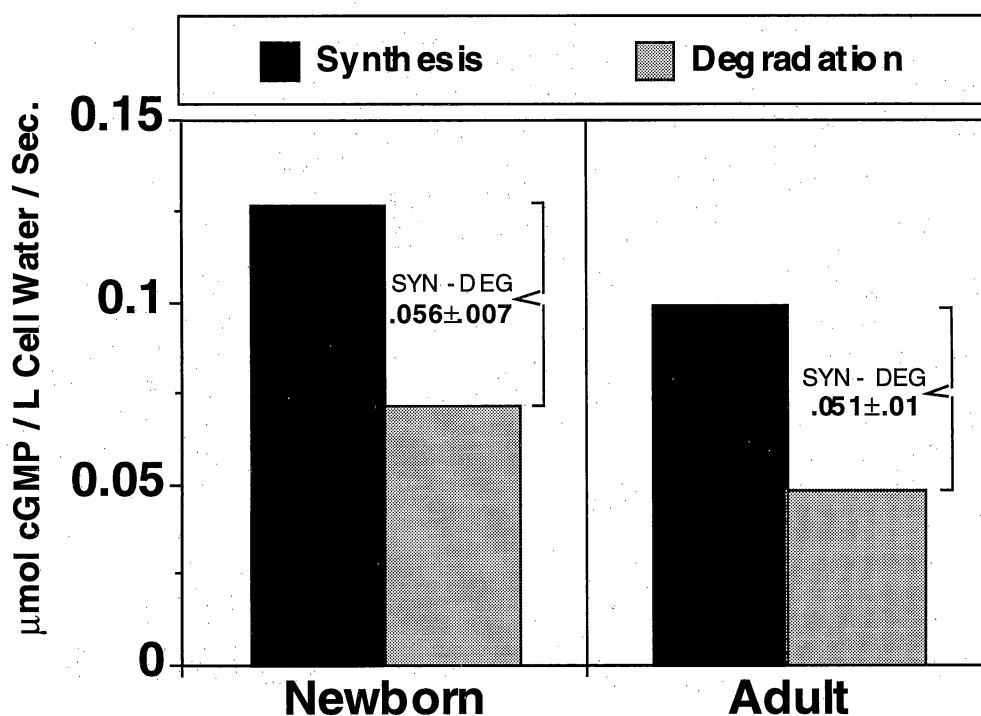

Figure 9: <u>The Differences Between Synthesis and Degradation of cGMP are Shown for Newborn and Adult Arteries.</u> All values are given as means N = 8 in the newborn and 13 in the adult.

Part II: Compartmentalization

The second section of this project was designed to determine if compartmentalization plays a role in PDE activity. I first hypothesized that compartmentalization may play a role in smooth muscle PDE activity when I considered the role it plays in GC activity. The subcellular distribution of GC in smooth muscle suggests that each enzyme isoform has a unique biological role. Whereas the soluble form might be situated to respond to intracellular cues and to react to diffusible substances such as NO, the membrane-bound enzyme seems poised to respond to extracellular messages. This elegant compartmentalization of cGMP synthesis undoubtedly has some physiological significance which is utterly destroyed in homogenized preparations [Lincoln, 1989]. Given the closely linked interrelationships between GC and PDE, the likelihood that PDE activity is also affected by compartmentalization is extremely high.

Further evidence for this possibility can be found in the PDE literature itself. Not only are the published values for PDE *Vmax* and *Km* varied, several of the PDE subtypes require co-factors that are known to be compartmentalized or subject to constant fluxes in availability. PDE type I is dependent upon both Ca^{2+} and calmodulin. PDE types II and III are either stimulated or inhibited by cGMP availability. In addition to co-factors, in intact tissue, PDE effectiveness is the result of one or more of the subtypes working in conjunction with one another. It is these considerations which lead me to believe the PDE activity was effected by compartmentalization.

Much to my surprise, none of the available data on PDE activity took into account the possible effect of compartmentalization. What I did find were

enzyme studies which methodically purified and isolated the different PDE families and isozymes, determined their various *Vmax's* and *Km's*, and then extrapolated those activities to physiological relevance. Although the identification, classification and characterization of the PDE enzymes is an important first step, accurately duplicating the necessary factors for optimum activity in a purified and isolated preparation, is virtually impossible. Unfortunately the only existing data regarding PDE activity is from preparations of this type.

It is this situation which led me to develop the *5-30 protocol*. As previously described, I conducted this study in both homogenized and isolated intact common carotid arteries from both age groups. I then compared the results from the two techniques, and found a dramatic difference between the two. The homogenate technique revealed nearly a two fold greater degradative capacity in the adult compared to the newborn. The *5-30 protocol* indicated the opposite. When I utilized the intact preparation of the *5-30 protocol*, the degradative capacity of PDE was greater in the newborn as opposed to the adult. When I compared the actual calculated rates of degradation between the two techniques, further discrepancies were observed. The rates of degradation for the newborn and adult in the homogenate preparation were 1.407 and 2.406 μmol cGMP $\cdot$ L cell water $^{-1} \cdot$ min $^{-1}$, respectively. When the estimated rates for the *5-30 protocol* are converted to the same units as the homogenate preparation, the rates for the newborn and adult become, 4.26 and 2.88 μmol cGMP $\cdot$ L cell water $^{-1} \cdot$ min $^{-1}$, respectively. Although the rates for the adult appear similar between the two techniques, the rates for the newborn are still very different. The rate similarity

between the techniques in the adult, can be easily explained by the relative differences in the cGMP substrate concentration of the two techniques. In the intact tissue, the PDE isozymes operate in an environment which never exceeds 2 μmol cGMP · L cell water $^{-1}$. In the homogenate preparation, the PDE isozymes are exposed to a cGMP substrate in excess of five time higher than that in the intact tissue. As previously stated, the calculated rate of degradation in the crude homogenate is actually an expression of *Vmax*. As such, any value for the *V* value can be altered depending upon the concentration of substrate. As the substrate concentration drops due to degradation, the rate <u>changes</u> and becomes non-linear. This phenomenon was observed during the actual homogenate experiments. When the cGMP substrate concentration began to approach physiological levels in the time points at the extreme end of the incubation, rates of degradation began to display a non-linear nature. This is a strong indication that the compartmentalization of the intact tissue allows the PDE isozymes to function at an optimum activity which can only be achieved in the homogenate preparation when the substrate is well in excess of physiological levels. With regards to the newborn, although the difference in the rates of degradation is maintained between the two techniques, this same explanation still applies.

Although this data is not conclusive, the difference in results between the crude homogenates and the *5-30 protocol,* strongly indicates that compartmentalization plays a physiologically important role in PDE activity.

As I developed and considered the results of the *5-30 protocol,* a practical application for my technique occurred to me. The *5-30 protocol* may prove to be extremely useful with the development of new PDE inhibitors.

Drugs such as *zaprinast* and *quazinone* which inhibit specific PDE isozymes, display a vast potential for clinical usage as tissue specific vasodiolaters. These drugs may be greatly superior to the traditional nitrovasodilators or beta-adrenergic blockers on two accounts; 1) many of the new PDE inhibitory drugs do not show the propensity for the development of tolerance [Polson, 1990], 2) due to the heterogeneity of PDE isozyme distribution, drugs with a selective PDE inhibition may be used to target specific tissue [Polson, 1990]. The selective inhibition of a single PDE isozyme within a target vascular bed, such as the cerebral circulation, would permit the subtle manipulation of intercellular cGMP levels and thus produce vasodilation exclusively in the target arteries. Unfortunately the development of any drug intended for human use requires extensive animal testing. Utilizing present techniques, large numbers of animals would be required to provide a sufficient quantity of tissue to test. Not only is the validity of these techniques in question, harvesting a sufficient quantity of tissue to test the efficacy of a drug targeted on the smaller vascular beds such as the cerebral arteries, would prove to be extremely difficult and costly. My technique has the potential of minimizing the number of animals normally required for testing, and readily allow for testing in specific vascular beds.

In conclusion the difference between synthesis/ degradation is similar in both newborn and adult, and it is the absolute levels of cGMP which differ. It is this mechanism that best explains the age-related differences in basal cGMP. The most important finding of this study is that the rates for PDE degradation are significantly different between the whole-unbroken and the homogenized preparations.

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