The Unexpected Evolution of Basic Science Studies about Cyclic Nucleotide Action into a Treatment for Erectile Dysfunction

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In these Reflections, I describe my perceived role in discoveries made in the cyclic nucleotide field that culminated in the advent of PDE5 inhibitors that treat erectile dysfunction, such as Viagra, Levitra, and Cialis. The discoveries emphasize the critical role of basic science, which often evolves in unpredictable and circuitous paths, in improving human health.

I grew up in two towns in western North Carolina, Franklin and Bryson City, both of which had populations of less than 3000. Most Americans refer to this region as Southern Appalachia, which is more widely known for its poverty than its scenic mountains and spectacular waterfalls. Perhaps the experiences with nature provide a good beginning to a scientific career; at least they provide inspiration for solving how nature works. I had five brothers and no sisters. My mother was a stay-at-home mom, and my father was a hospital laboratory technician and did some medical work, including house calls, although he only completed two years at Emory Medical School. I owe my father significant credit for inspiring me to pursue a scientific career later on. In high school, I succeeded in several sports, especially football, and was awarded a scholarship to attend Tennessee Technological University (Fig. 1). I played in the Tangerine Bowl in 1961 and was co-captain of the team in 1962. We won the Ohio Valley Conference championship three of my four college years. After finishing the three-year pre-medicine program, I switched my major to biology and was able to play football for one more season. It was during that year that I was introduced to a scientific career through the influence of one of my biology professors, Dr. William Downs, who was a colleague of Charles Rawlinson (Rollo) Park, chairman of the Department of Physiology at Vanderbilt University.

A scientific career for me would have been improbable, particularly because of financial considerations, were it not for a highly significant world event. The first man-made satellite, Sputnik, was launched by the Soviet Union in 1957. Sputnik unleashed the Space Race between the United States and Soviet Union and led to the moon landings and many other achievements in space. It also ushered in a scientific renaissance within the American public and government during the Kennedy and Johnson presidential administrations. Among many new programs, fellowships were made available to prepare scientists for advanced degrees not only in space technology, but in practically all of the sciences, including the biosciences. The years that followed were fruitful for the scientific community and inspired those who had an appreciation for and a belief in the
improvement of the quality of life through scientific advances. Due in no small part to Sputnik, I received a training grant for graduate level research in physiology at Vanderbilt University in 1963.

I chose Rollo Park as my Ph.D. thesis advisor at Vanderbilt (Fig. 2). He is a very decent and dignified man who is exceptionally wise, patient, and scientifically competent. After his undergraduate education at Harvard University, Rollo received his medical degree at Johns Hopkins University and then did research training at Washington University in St. Louis under Carl Cori. Rollo went directly from his postdoctoral studies under Cori to chairman of the Department of Physiology at Vanderbilt, a remarkable career ascent! I remember fondly many of our scientific and political discussions, as well as our outdoor excursions together in the Tennessee hills and streams. At the time of this writing, Rollo, at 98 years of age, resides with his wife, Janey, in a Nashville suburb.

Earl Sutherland, who had discovered cAMP in the late 1950s, was a longtime friend of Rollo Park since they had both done postdoctoral studies in the Cori laboratory at the same time. Sutherland was recruited by Rollo to Vanderbilt’s Department of Physiology in 1963. This event profoundly affected my scientific career. At that time, Sutherland and his collaborators, who included Theodore (Ted) Rall, Reginald (Bill) Butcher, Joel Hardman, G. Alan (Al) Robison, Rollo Park, Grant Liddle, Howard Morgan, and John Exton among many others, had already demonstrated a role for cAMP in the regulation of a few physiological processes, particularly for epinephrine stimulation of glycogen breakdown in the liver. Following their discovery, they fully realized that cAMP was likely involved in the regulation of many physiological processes (1). This subject was the center of attention within our department and throughout the Vanderbilt University Medical Center. Discussions in the hallways, seminars, workshops, and other gatherings were invariably lively and often centered on the actions of cyclic nucleotides. I remember vividly the heated debates and shouting matches that took place during the departmental and interdepartmental seminars, at Gordon Conferences, and at national meetings, which

FIGURE 1. Jackie Corbin playing football for Tennessee Tech in 1961.

FIGURE 2. Rollo Park, chairman of the Department of Physiology at Vanderbilt University in the 1950s–1980s.
Early History of Research on Cyclic Nucleotide Action

Most of the early investigations of cyclic nucleotides were centered on the cyclic nucleotides themselves and the roles they played in body tissues (1). Some of this work was done by Sutherland at Case Western Reserve University before he arrived at Vanderbilt. Sutherland and his colleague Ted Rall discovered cAMP as a heat-stable factor produced in cell-free liver homogenates by the addition of adrenalin or glucagon. cAMP caused the conversion of glycogen phosphorylase to a phosphorylated active form when added to supernatant fractions of the homogenates. In the intact organism, elevation of the cAMP level in the liver resulted in increased glycogen breakdown to glucose during physiological stresses, such as exercise and starvation. Together with David Lipkin at Washington University in St. Louis, who had worked previously on the Manhattan atomic bomb project in Los Alamos, New Mexico, they identified the crystallized factor chemically as a novel compound, cAMP. Sutherland showed that cAMP is synthesized by the enzyme “adenyl” cyclase (adenyl cyclase) and that it is inactivated by the hydrolysis of the high-energy cyclic phosphate ring to form 5′-AMP by a “cyclic 3,5-AMP-inactivation enzyme” activity from heart, brain, or liver extracts, which was termed a phosphodiesterase (PDE) activity (1). Caffeine, a methylxanthine, was found to inhibit the PDE, which was the first demonstration of a mechanistic effect of caffeine on an enzyme. As discussed below, this was a critical milestone leading to our research that resulted in the advent of PDE5 inhibitors for treatment of erectile dysfunction.

At Vanderbilt, much of the effort of the Sutherland group emphasized the broad regulatory functions of cAMP. Some of these included adrenalin stimulation of liver glycogenolysis and gluconeogenesis, as well as of adipose tissue lipolysis. During the 1960s, numerous laboratories were demonstrating myriad roles for cAMP in mediating the effects of many hormones (1). On the basis of the intracellular/extracellular locations of the steps within the hormonal pathways and abundant regulatory roles for cAMP established at that time, Sutherland proposed the “second messenger hypothesis.” According to this concept, a signal (e.g. a hormone (first messenger) derived from one cell type or organ) interacts with the exterior of another cell to elicit generation of cAMP (second messenger). By this process, the first messenger transfers the message to the inside of a target cell, thereby altering metabolism and other physiological processes.

Sutherland was awarded the Nobel Prize in Physiology or Medicine in 1971. In the week of the Nobel announcement, I recall that a member of the Nashville press interviewed him and asked for advice for young people on how to win a Nobel Prize. He said that, first of all, one must be lucky, and, second of all, one must recognize the luck when it comes!

1 The abbreviations used are: PDE, phosphodiesterase; PKA, protein kinase A (cAMP-dependent protein kinase); PKG, protein kinase G (cGMP-dependent protein kinase); IBMX, 3-isobutyl-1-methylxanthine.

cGMP

Although cAMP was the only established second messenger during the early 1960s, Sutherland and his co-workers stated that it seemed “possible or even likely that other second messengers will be discovered” (1). This prediction turned out to be accurate for many signaling pathways, even though some exhibited slight deviations from the original theory. For example, several first messengers were found to traverse the cell membranes to generate second messengers within cells.

In 1963, T. D. Price and his co-workers at Columbia University discovered cGMP in urine as a radioactive compound following the injection of radioactive inorganic phosphate into rats (2). Subsequently, cGMP was shown to be present in all tissues tested (1). However, many attempts during the 1960s to demonstrate hormonal alteration of the level of cGMP in tissues were unsuccessful, even though rapid turnover of this nucleotide was well documented (1). “Guanyl” cyclase (guanylyl cyclase), which catalyzes the synthesis of cGMP from GTP, was discovered in 1969 (3), and by 1970, crude preparations of PDEs were shown to catalyze breakdown of cGMP as well as cAMP (4).

During the 1970s, the groups of Günter Schultz with Joel Hardman at Vanderbilt and the University of Heidelberg, Germany, and Ferid Murad at the University of Virginia showed that treatment of several smooth muscle tissues with compounds that were capable of generating nitric oxide causes elevation of cGMP levels and concomitant relaxation of those tissues (5, 6). This was the first indication of a possible physiological role for cGMP, even though it was known that cAMP elevation would also cause smooth muscle relaxation. The fact that blood vessel walls were known to be rich in smooth muscle cells suggested that cGMP could play an important role in the regulation of blood flow and pressure, but there was strong bias that cAMP signaling was more important in this pro-
cess, given the proven role of cAMP in so many other biological processes. The role of cGMP in processes other than visual transduction was also not established. It was also known during the 1970s that penile tissue is rich in smooth muscle and that relaxation of this muscle plays a critical role in penile erection (7).

Cyclic Nucleotide-dependent Protein Kinases

Sutherland and his collaborators demonstrated that cAMP is the mediator of many hormonal signals, but they did not pursue, to a significant extent, the molecular mechanisms of its action or that of cGMP action on body functions. During the 1960s, the groups of Joe Larner at the University of Virginia (8) and Edwin Krebs at the University of Washington (9) did pioneering work in elucidating the biochemical mechanism of action of cAMP in inhibiting glycogen synthesis and stimulating glycogen breakdown, respectively, in skeletal muscle. They deduced that these effects are mediated by a protein kinase that is stimulated by cAMP, which Krebs named cAMP-dependent protein kinase (protein kinase A (PKA)), implying that it phosphorylates more than a single protein.

I conducted my Vanderbilt thesis project (10) on the anti-lipolytic effect of insulin in adipose tissue. Using an isolated fat cell preparation that had recently been described by Martin Rodbell at the National Institutes of Health (11), I demonstrated that insulin inhibits adrenaline-stimulated lipolysis by lowering cAMP and that this effect, or the effect of adrenaline to stimulate lipolysis, occurs within a very narrow 2–3-fold range of concentrations of cAMP. Because I found that insulin blocks the lipolytic effect of adrenaline or exogenous cAMP, but not that of exogenous dibutyryl-cAMP, which is resistant to breakdown by a PDE, I proposed that insulin acts by stimulating the activity of a PDE to lower cAMP. Some of this work was done in collaboration with Bill Butcher and Sam Sneyd. At that time, Sneyd was doing postdoctoral studies at Vanderbilt on cAMP and insulin action with my mentor, Rollo Park. Somewhat later, Sneyd returned to his native University of Otago in New Zealand and, along with his student David Loten, who had also studied at Vanderbilt as a postdoctoral fellow, showed using liver slices that insulin does indeed stimulate a particular "low K_m" PDE, later named PDE3 (12, 13). Today, we recognize PDE3 as a critical mediator of insulin action in the liver and adipose tissue during starvation, exercise, and obesity.

Having completed my doctoral thesis project at Vanderbilt, I had a strong desire to continue studies on the mechanism of action of cAMP in adipose tissue. Rollo Park wisely advised me to do postdoctoral studies with Edwin Krebs (Fig. 3). Rollo thought that experience in the Krebs’ laboratory would have the added advantage of broadening my knowledge base and enhancing my career experiences because it would be more biochemical in nature and would involve learning enzyme purification procedures. Krebs agreed wholeheartedly to carry out the adipose tissue project. Despite Krebs moving to the University of California, Davis, just before my arrival, I was not deterred, although I did have some inhibitions about living outside The South for the first time. While at Davis, my wife, Ann, gave birth to our only child, Amy, in 1970.

While working in the Krebs’ laboratory, I demonstrated that PKA mediates the ATP-dependent cAMP activation of lipolysis by hormone-sensitive lipase stimulation (13). Another member of the Krebs’ group, Tom Soderling (14), showed simultaneously that the same PKA mediates phosphorylation and inactivation of liver glycogen synthase; this had also been shown by Joe Larner earlier, as mentioned above. Taken together with the previous finding that PKA mediates cAMP-stimulated glycogenolysis, these observations provided strong evidence that PKA mediates many effects of cAMP elevation in tissues. In fact, during the 1970s, many proteins were shown to be phosphorylated and modulated by this enzyme. It seemed unconventional at the time to suggest that a single enzyme could carry out the same catalytic effect on so many diverse substrates, especially because scientists had become indoctrinated about the reasons and mechanisms for enzyme specificity, yet it proved to be the case. The dogma that PKA...
mediated all of the myriad effects of cAMP in mammals prevailed for many years until other cAMP mediators were discovered (15).

In collaboration with Margaret Brostrom, Charles Brostrom, and Erwin Reimann in the Krebs’ laboratory, I carried out enzyme work to establish that PKA is composed of a catalytic subunit that is inhibited by an associated regulatory subunit (16). Subsequently, I established that the inhibitory mechanism of the regulatory subunit was conferred by a competitive substrate mechanism within its structure (17). At the time, this was a novel inhibitor mechanism and has often been referred to as a pseudo-substrate mechanism. Subsequent studies established that this is a relatively common inhibitory mechanism for many other protein kinases. In the case of PKA, cAMP binding to the regulatory subunit leads to dissociation of the regulatory and catalytic subunits, thereby resulting in removal of inhibition of the catalytic subunit and followed by catalysis of the phosphorylation reaction. In those days, it was considered very unusual for an enzyme to be activated by the dissociation of its subunits.

In 1971, I returned to Vanderbilt (where I remain to this day) to join the faculty of my old Department of Physiology, which was still chaired by Rollo Park. The department is now known as the Department of Molecular Physiology and Biophysics. My first significant achievement after my arrival was to show that PKA exhibits two major isozymic forms, which I named types I and II (18). I found that type II is bound primarily to subcellular particles of heart homogenates, whereas type I is mainly soluble (19). The bound holoenzyme adheres to the particles through its regulatory subunit. When cAMP is elevated and binds to the regulatory subunit, the catalytic subunit dissociates from the regulatory subunit and from the particles. I proposed a model for cells in which the bound holoenzyme is compartmentalized, or anchored, near its substrate(s). This would be an efficient mechanism for phosphorylation and modulation of a substrate when the catalytic subunit is activated by its dissociation from the regulatory subunit following cAMP elevation. On the basis of the characteristics of its particle binding, I suggested that the regulatory subunit is anchored by binding to other particle protein(s) rather than being an intrinsic membrane protein. In subsequent years, the membrane proteins to which the regulatory subunit is anchored were identified by others as a family of A-kinase anchoring proteins (20, 21).

Tom Lincoln arrived at Vanderbilt as my first postdoctoral fellow in 1974. He grew up mainly in Decatur, Alabama, and received his Ph.D. degree from the University of Tennessee. Tom had contacted Rollo Park regarding post-doctoral study, and Rollo then directed him to my laboratory. In 1970, the group of Paul Greengard (Nobel laureate in 2000) discovered cGMP-dependent protein kinase (PKG) in lobster tail muscle (22, 23). Because very few biochemical studies on PKG had been done by that time, Tom and I felt that some significant findings concerning cGMP action could be made by carrying out extensive enzyme purification and kinetic studies on PKG much as I had already done on PKA. Greengard’s group, working with lobster tail tissue, had reported that PKG, like PKA, is composed of a cGMP-binding regulatory subunit and a catalytic subunit, which dissociated from each other upon the addition of cGMP when examined in a crude preparation. In his initial work in my laboratory, Tom identified a specific PKG activity in mammalian tissues. On the basis of earlier experience with PKA, Tom and I were biased that our purified lung PKG should contain separate regulatory and catalytic subunits that dissociate from each other during cGMP binding to a regulatory subunit; however, Tom was unable to establish this process. We reasoned that the regulatory and catalytic components of PKG, unlike those of PKA, are located on a single subunit and that cGMP activates the lung PKG by causing a conformational change rather than by causing a physical dissociation of subunits (23). We thought that the lobster tail PKG actually has a similar structure and mechanism of activation as the lung PKG, but that regulatory and catalytic components on the same protein chain of the lobster tail PKG are cleaved into separate fragments by contaminating proteases during activation of the enzyme by cGMP. Likewise, Gordon Gill at the University of California, San Diego, concluded that PKG does not contain separate regulatory and catalytic subunits (24), and from their studies of PKG from silkworm, Yasutomi Nishizuka’s group at Kobe University in Japan independently drew the same conclusion about PKG (25). Although this proved to be the correct mechanism, it turns out that PKG is more common in its activation mechanism and that PKA is more unusual in comparison with other enzymes. Most enzymes do not dissociate into free subunits during activation.

Despite the apparent differences in subunit composition and structure, both PKA and PKG proved to be remarkably similar in other respects. For example, both are inhibited by pseudo-substrate mechanisms and activated by binding of cyclic nucleotides to their regulatory regions/subunits; crude structural properties are similar; and importantly, both kinases recognize similar primary structural cues in substrate proteins to catalyze very specific serine/threonine phosphorylation. In 1977, we pro-
posed that PKA and PKG are homologous proteins and predicted that the protein kinase family might be a much larger one (26). In 1977, only a handful of protein kinases were known; protein kinase C, calcium/calmodulin-dependent protein kinase II, and the tyrosine kinases were yet to be discovered.

Cyclic Nucleotide-binding Proteins and the Discovery of PDE5

The investigations that I carried out with my collaborators on PKA and PKG eventually led to the discovery of PDE5, the receptor for Viagra, Levitra, Cialis, and other commercial PDE5 inhibitors.

While I was doing my postdoctoral studies in the laboratory of Edwin Krebs, the regulatory subunit of PKA was being studied not only for its inhibitory activity toward the catalytic subunit of the enzyme, but also for its cAMP-binding activity. When I returned to Vanderbilt, I optimized a [3H]cAMP-binding assay for the regulatory subunit (27). Using this assay, a [3H]cAMP-binding stoichiometry of two cAMP molecules/regulatory subunit was established. These two binding sites were demonstrated to be quite different from each other even though both were important for enzyme activation (12). The [3H]cGMP-binding assay for the regulatory component of PKG was also optimized, and I showed that this kinase also contains two binding sites/subunit, albeit both are selective for cGMP over cAMP (28).

In the mid-1970s, the dogma was widespread that PKA and PKG mediated all of the mammalian physiological effects of cAMP and cGMP, respectively. Tom Lincoln and I believed that there could be other mediators. In 1970, the group of Zubay (29) at Columbia University had reported the presence of a non-protein kinase cAMP receptor in Escherichia coli named the catabolite activator protein, but the presence of this protein or any cAMP or cGMP receptor, other than PKA and PKG, had not been demonstrated in mammals. The development of stoichiometric cyclic nucleotide-binding assays offered an opportunity to search for such proteins that specifically bind cAMP or cGMP. Believing that we were among the few scientists in the world vigorously pursuing cGMP action at that time, we decided to search for cGMP-binding proteins instead of cAMP-binding proteins. We made homogenates from several rat tissues, centrifuged them, and then subjected the supernatants to DEAE-cellulose chromatography to obtain a crude separation of the soluble proteins from the various tissues (30). The [3H]cGMP-binding assay of the fractions obtained by chromatography of several tissue extracts revealed a PKG peak in its typical elution position as expected. Importantly, some of the tissue extracts, such as that from the lung, exhibited an additional peak of [3H]cGMP-binding activity eluting ahead of the PKG peak. This fraction was clearly highly specific for cGMP over cAMP, was not a protein kinase, and possessed other physical and kinetic properties indicating that it was unique. Our report of these studies was the first to indicate the presence of a mammalian cAMP or cGMP receptor other than the cyclic nucleotide-dependent protein kinases. Among several kinetic features of this newly discovered protein was its stimulation of [3H]cGMP binding by the PDE inhibitor 3-isobutyl-1-methylxanthine (IBMX). Although this finding suggested that it could be a PDE, more definitive proof was needed because some thought that this activity could be due to a breakdown product of PKG or that the “stimulation” by IBMX was simply due to protection of the substrate cGMP from breakdown. Other interesting features surrounding this protein, including important medical breakthroughs, would be forthcoming (Fig. 4).

A key event in uncovering the identity, function, and eventual medical use of PDE5 inhibitors such as Viagra was the return of Sharron Francis as a faculty member in the Vanderbilt Department of Physiology in 1975. Sharron and I had experienced similar prescientific and early scientific careers during the 1940s–1960s. Sharron grew up in eastern and central Kentucky and I in the Appalachian Mountains of western North Carolina. Sharron did her undergraduate work at Western Kentucky University and I at Tennessee Tech. As undergraduates, both of us had majored in biology. Both of us had received post-Sputnik government training grants to do Ph.D. thesis research in the Vanderbilt Department of Physiology. Both of us had been exposed to the cyclic nucleotide research done at Vanderbilt during the 1960s. I had chosen Rollo Park as my thesis advisor, and Sharron had chosen Rollo’s wife, Janey. Sharron had done postdoctoral studies involving protein structure and regulatory processes in two outstanding laboratories, those of Herman Eisen at Washington University and of Earl Stadtman at the National Heart, Lung, and Blood Institute (Fig. 5).

In 1977, Sharron joined Tom and me in a collaboration to determine the function of the newly discovered cGMP-binding protein. In those days, very few consensus amino acid or nucleotide sequences of functional domains for any protein family were available to guide us. Biochemists ordinarily used the approach of purifying a protein extensively and establishing if a particular functional property was retained in that protein. Because of the stimulatory effect of IBMX on the cGMP-binding activity that Tom
had observed, we believed that the protein could be a PDE, but many people doubted that interpretation. Therefore, we tested whether or not the IBMX-stimulated cGMP-binding activity correlated with PDE activity in the chromatographic profiles during several steps of purification of the protein (31). Using rat lung as starting material, Shar-ron spent more than a year in carefully developing conventional purification steps. She found that the two activities do indeed co-chromatograph precisely. Moreover, the stimulatory effect of IBMX on \(^{3}\text{H}\text{cGMP}\)-binding activity, which is also obtained using other PDE inhibitors, such as papaverine, theophylline, and caffeine, is retained in the purified protein. The PDE was found to be highly specific for cGMP over cAMP as a substrate. Some predicted that the \(^{3}\text{H}\text{cGMP}\)-binding activity of the enzyme was conferred by its substrate site and even perhaps a modified substrate site. However, because PDE inhibitors were known to be competitive with cGMP for the substrate site of PDEs, it would be predicted that PDE inhibitors should be inhibitory, not stimulatory, toward \(^{3}\text{H}\text{cGMP}\) binding. Moreover, binding of \(^{3}\text{H}\text{cGMP}\) to the PDE substrate site did not have sufficient affinity to be detected by our \(^{3}\text{H}\text{cGMP}\)-binding assay. The combined results suggested that the protein possesses two different kinds of sites for cGMP action, a catalytic site for breakdown of cGMP and a binding site for regulation by cGMP. This was confirmed by the findings that heat or trypsin treatment of the purified protein destroys PDE activity but stimulates cGMP-binding activity and that certain cGMP analogs inhibit PDE activity but not cGMP-binding activity. The catalytic site and cGMP-binding site are non-homologous (32). This PDE, which would ultimately be the target for PDE5 inhibitors in treating erectile dysfunction and pulmonary hypertension, thus represented the first recognized mammalian cyclic nucleotide receptor other than PKA and PKG. We named it the cGMP binding protein-PDE, which was later changed to PDE5 in the 1990s.

**Physical and Functional Characterization of the Newly Discovered cGMP-binding PDE5**

Tom Lincoln finished his postdoctoral studies in my laboratory in 1979 and continued to work on PKG in smooth muscle. His laboratory at the University of South
Carolina was the first to propose that cGMP lowers intracellular calcium levels in smooth muscle to induce relaxation (33). From this point, Sharron and I continued our close collaborative investigations of PDE5. We shared all of the laboratory functions about equally, including mentorships, writing grants and papers, and presenting talks. We discussed experimental results and ideas daily. In addition to inserting more enjoyment into our research experiences, this arrangement provided enormous advantages for our investigations. I highly recommend a similar organization to developing scientists. There are many examples of such successful collaborations in the scientific world (Fig. 6).

PDE5 potentially represented a new direction for research on cyclic nucleotide action. The function(s) of the cGMP-binding site of the enzyme were unknown. Biochemical studies could reveal interesting features not only about the regulation of the enzyme, but also about its catalytic properties and physiological roles. The literature already contained implications for important roles for cGMP in smooth muscle regulation, particularly for cardiovascular diseases such as hypertension. We were well aware of the potentiating effect of PDE inhibitors to raise the tissue levels of cAMP or cGMP by inhibiting PDEs. For example, such an effect would raise the cGMP level in arterial smooth muscle, relax the muscle, and lower blood pressure, which suggested that a cGMP-PDE inhibitor could be used to treat hypertension.

Having worked out the arduous process of purifying PDE5 from rat lung, we decided to switch from rat lung to bovine lung as the tissue source to obtain larger quantities of PDE5 for molecular studies. Through much diligent work by Melissa Thomas, Linda McAllister, and Janet Colbran, the first amino acid sequences were obtained, and this led to the cloning of the cDNA of the enzyme via collaboration with Joseph Beavo’s laboratory at the University of Washington (32, 34, 35). Melissa Thomas discovered that PKG phosphorylates the dimeric enzyme at a single serine residue in its regulatory domain and that this modification stimulates the PDE catalytic activity (26). Working together with our students Mitsi Blount and Emmanuel Bessay, we uncovered several other regulatory features of the enzyme. Phosphorylation of PDE5 stimulates cGMP binding to the regulatory site (36), which stimulates the catalytic site of PDE5, as well as the phosphorylation of the enzyme (37). Finally, we found that when a substrate binds at the substrate site, it stimulates both cGMP binding at the regulatory site and phosphorylation of the enzyme. These findings together indicated that elevation of cGMP in cells causes a concerted feedback response to activate PDE5 and results in lowering of the cGMP level, a classic negative feedback effect.

**Regulation of Smooth Muscle Function, Blood Pressure, and Blood Flow**

Because of their critical roles in species survival, blood flow and blood pressure are highly regulated processes (Fig. 7). A plethora of mechanisms exist for their control. One salient mechanism targets the smooth muscle cells in the walls of blood vessels. In general, contraction of these smooth muscles constricts arteries, decreasing downstream blood flow and increasing upstream blood pressure. Conversely, relaxation of these smooth muscles dilates arteries, increasing downstream blood flow and decreasing upstream blood pressure. The sympathetic and parasympathetic nervous systems are prominent regula-
tors of contraction/relaxation of blood vessel smooth muscles. Common neurotransmitters for sympathetic nerves are adrenalin and noradrenalin, thereby referred to as “adrenergic.” Parasympathetic nerves commonly employ acetylcholine; thus, they are referred to as “cholinergic.” In the early 1980s, the dilation of penile cavernosal smooth muscle to produce erections was thought to be mediated by parasympathetic nerve stimulation.

Up until the mid-1980s, either cAMP or cGMP elevation was known to produce smooth muscle relaxation, but the mechanism for each nucleotide was unknown, even though many workers in the field believed that PKA or PKG mediated the respective cyclic nucleotide effect. However, we found that although either cAMP or cGMP analogs caused relaxation of vascular and tracheal smooth muscles, only those analogs that activated PKG relaxed the muscles (some cAMP analogs activated PKG) (38). We could find no evidence for PKA involvement in mediating the relaxation effects. We suggested that cAMP produces its physiological relaxation effect by “cross-activation” of PKG (39). Our attention centered on the cGMP pathway, and because we had shown that smooth muscle (and lung tissue) was rich in both PKG and PDE5, we surmised that coordinated activation/inhibition of these respective proteins would lead to development of a particularly potent vasodilator or bronchodilator. On this premise, we devised the idea that perhaps a drug, such as a PDE5-resistant cGMP analog, could be developed that would serve as a dual-acting compound to activate PKG and inhibit PDE5, thus substantially elevating cGMP to produce vasodilation or bronchodilation for treating certain diseases.

**Penile Erection**

It was known by the early 1980s that the penis contains a paired cylindrical structure termed the corpus cavernosum, which is primarily responsible for penile erection (Fig. 8) (7). This vascularized spongy structure is highly enriched in smooth muscle cells that surround blood vessels and cavities known as sinusoids. It was known that sexual arousal in men specifically induces parasympathetic nerve stimulation in penile tissue and was believed to be non-cholinergic and non-adrenergic. However, the neurotransmitter had not been identified. The nerve stimulation brings about relaxation of the penile smooth muscle cells, leading to increased blood inflow to and expansion of the sinusoids, which compresses the veins to reduce outflow of blood from the sinusoids. Together, these processes cause an ~8-fold increase in penile blood volume, resulting in a full erection due to the swelling and rigidity of the penis.

**Nitric Oxide and Its Role in Stimulating Penile Erection**

Before 1990, the organic cause(s) of erectile dysfunction were largely unknown, and psychotherapy was a method of choice for treating this condition. Penile implants, vacuum constriction devices, and surgeries also were used, although understandably, most patients...
were not attracted to these methods. Direct penile administration of prostaglandin E₁ and other agents was sometimes effective, but was also unacceptable to most patients. Various oral treatments were in use, but they were generally ineffective.

In the 1980s, nitric oxide was discovered as an important signaling molecule. The groups of Ferid Murad at the University of Texas, Robert Furchgott at the State University of New York Health Science Center, and Louis Ignarro at UCLA received the Nobel Prize in 1998 for their work on this subject. In 1990, the Ignarro group reported that nitric oxide-induced cGMP elevation in penile smooth muscle mediates stimulation of penile erection in rabbits (40). Two sources of nitric oxide came to be recognized in this process: parasympathetic nerves, which mediate the effects of sexual arousal; and vascular endothelial cells, which mediate the effects of local penile factors. The idea was emerging that erectile dysfunction was caused primarily by insufficient nitric oxide release within the penis to adequately elevate cGMP in the vascular smooth muscle cells of this tissue (41). If so, we thought that a PDE5 inhibitor, which would elevate cGMP by preventing its breakdown, would be therapeutic for men with this condition.

The general principle of synergism (potentiation) was established in the early 1960s by Rall and Sutherland. They showed in liver preparations that inhibition of PDE-catalyzed breakdown of cAMP by a PDE inhibitor (caffeine in this case) produces a much bigger elevation in cAMP and thus a bigger downstream physiological response in the presence of a stimulator of adenylyl cyclase-catalyzed cAMP synthesis, such as epinephrine or glucagon. Synergism works for the cGMP pathway as well as for the cAMP pathway. Therefore, a PDE5 inhibitor would have its greatest effect in tissues that already have a stimulated rate of cGMP synthesis. In the case of penile erection, this stimulated rate is provided by sexual arousal. The arousal causes increased cGMP synthesis, and the PDE5 inhibitor causes decreased cGMP breakdown, which synergistically and selectively elevates the penile smooth muscle cGMP level and leads to the relaxation of the muscle and penile erection. Viagra and the other commercial PDE5 inhibitors have very little effect unless a man is sexually aroused. The synergism provides a targeting effect of a PDE5 inhibitor to cause penile erection in the absence of significant side effects because cGMP synthesis in body tissues, other than penile smooth muscle, is not activated by sexual arousal. As Ken Murray at SmithKline Beecham Pharmaceuticals (now GlaxoSmithKline) stated in a review in 1993 (42), “there could be considerable value in a therapeutic agent that has little activity in its own right, but potentiates the effects of endogenous mediators.”
After the advent of Viagra in 1998, I got numerous invitations to present lectures on treatment of erectile dysfunction. In addition to universities and international meetings, I also enjoyed giving PowerPoint lectures to rural Tennessee doctors and nurses, senior citizen groups, service organizations, and schools. One memorable experience followed a lecture I gave to a small-town middle school group. Part of my presentation included the slide depicted in Fig. 8, which is a picture of the statue of David by Michelangelo with an inset showing the cross-section of a penis. Although this picture and its description may have been well received by the students, this was not the case for many of the teachers, parents, townspeople, and local politicians, who were in attendance. Before I arrived home after a two-hour drive from the school, the Nashville television channels presented interviews with some of those irate citizens on the evening news. The slide of Fig. 8 was used as a backdrop by one of the channels during the presentation, albeit with the penis covered by a black rectangle. The local reporters had visited the students in my laboratory, who gave them the requested slide before I returned from my trip. News of this embarrassing incident reached many of my colleagues around the world. Several Europeans were particularly amused by the implication that the statue of David was a pornographic image.

The Vanderbilt Lawsuit

In the late 1980s, Sharron and I pursued the synthesis and study of cGMP analogs to determine whether, by appending certain chemical groups, the resulting analogs might more effectively activate PKG. Through the phosphorylation of other proteins, the enzyme reduces the level of cellular calcium, resulting in relaxation of the smooth muscle cell. In 1989, we were awarded a three-year Glaxo Cardiovascular Discovery Grant to further pursue our research. During 1991, the final year of the grant, Glaxo encouraged us to emphasize the development of PDE5 inhibitors over PKG activators because cGMP analogs break down in the digestive system before reaching targeted smooth muscle cells and thus were not good candidates for orally administered drugs. PDE5 inhibitors act by competing with the enzyme substrate cGMP, so these inhibitors have some structural similarity to cGMP. On the basis of the knowledge of PDE5 gained from our studies of cGMP analogs, Sharron and I, along with Sekhar Konjeti (known as Raja), who was an excellent chemist and had joined us as a postdoctoral fellow to synthesize compounds in connection with the 1989 grant, theorized that more potent PDE5 inhibitors could be created by attaching hydrophobic groups, such as a phenyl ring, with additional electron-donating groups appended to the ring, in positions where they could bind to the same site on the PDE5 molecule as does the ribose phosphate moiety of cGMP. Moreover, because our research suggested that cGMP bound more tightly to the catalytic site on PDE5 when the ribose phosphate moiety was in the anti-position in relation to the guanine moiety, we further theorized that inhibitor potency would be enhanced by appending the phenyl ring at the 8-position on the six-member/five-member ring scaffold, as opposed to the 9-position, where the ribose phosphate moiety of cGMP joins its guanine moiety. Applying these theories to IBMX, we conceived 8-(4-OH-phenylthio)-IBMX (Scheme 1). Tests showed that 8-(4-OH-phenylthio)-IBMX was 160 times more potent than IBMX in inhibiting PDE5 and six times more potent than zaprinast, which was the most potent inhibitor of PDE5 then known.

On November 12, 1991, we sent a letter to the Vanderbilt Department of Technology Transfer explaining the potential commercial applications for our PDE5 inhibitors. Having read the Ignarro report concerning cGMP involvement in penile erection (40), we explained that the inhibitors could be useful to treat "male impotence" or "any malady that involves smooth muscle." To our knowledge, this was the first written mention that PDE5 inhibitors could be used to treat this condition.

During a telephone conversation in late December 1991, I discussed our success using PDE5 inhibitors with the head of the Glaxo Group Research’s cardiovascular research management committee. He requested that I send him a letter summarizing our research and a proposal for a possible second grant from Glaxo to fund further research. In the letter we sent on January 3, 1992, years before inhibitors such as sildenafil (Viagra) were tested for that purpose, we pointed out that PDE5 "is present in high concentrations in vascular smooth muscle," such as the penile corpus cavernosum, and that PDE5 inhibitors could be used to treat "male impotence" in addition to other diseases. We also described our theories for designing PDE inhibitors...
inhibitors and the significant increases in potency that we had achieved. However, we identified our inhibitors only as IBMX analogs.

Our letter prompted the Glaxo representative to fly from London to meet Sharron, Raja, and me at Vanderbilt on February 3, 1992, to discuss a formal Glaxo grant application for us to conduct further research on cGMP analogs and PDE5 inhibitors. He told us that we should submit a research proposal with more detail describing our experimental design. We did so on February 24, 1992. The proposal repeated our proposition that newly synthesized PDE5 inhibitors, based on our scheme of appending favorable chemical groups to IBMX, could be used to treat male impotence, later referred to as erectile dysfunction. It also disclosed and outlined the chemical structure of our potent inhibitor, 8-(4-OH-phenylthio)-IBMX, which we had synthesized in November 1991.

On April 8, 1992, the head of Glaxo Group Research’s cardiovascular research management committee sent copies of our research proposal to a number of Glaxo scientists, including the lead chemist at a Glaxo facility near Paris, France, who was leading a team tasked with developing new PDE5 inhibitors. On April 23, 1992, members of that Glaxo team tested 29 compounds (including, as a control, the known PDE5 inhibitor zaprinast) for PDE5 inhibition, each of which included a six-member/five-member ring. Twenty-six of the 29 compounds included some substitution at the 8-position of the six-member/five-member ring scaffold. Eleven of the 29 compounds tested on April 23 contained structural features found in our inhibitor, 8-(4-OH-phenylthio)-IBMX, which we had synthesized in November 1991.

Although we ultimately entered a second research agreement with Glaxo and shared the continuing results of our PDE5 inhibitor research directly with members of Glaxo’s PDE5 inhibitor team through 1994, we were unaware of these developments. It was not until 2003, after the publication of The Discovery of Tadalafil (43, 44), that we came to believe that our work had contributed to that discovery. This belief was based on a number of factors, including the reality that we had actively interacted with almost all the Glaxo scientists listed as authors on the papers and that the time frame described for the development of tadalafil coincided closely with the time frame in which we were collaborating with this group of scientists.

In 2005, Vanderbilt University filed a civil action against ICOS Corporation, to which Glaxo had assigned the rights to the patents for compounds, including tadalafil, and their use for the treatment of erectile dysfunction, seeking to correct the patents by adding Sharron, Raja, and me as additional joint inventors. The case came to trial in January 2008.

Prior to the trial, ICOS maintained that, as described in The Discovery of Tadalafil (43, 44), the Glaxo scientists were led to pursue the first compound leading to the discovery of tadalafil by a scientific paper that had reported...
that \( \beta \)-carboline analogs had been shown to be PDE5 inhibitors, albeit very weak, and that tests Glaxo performed on such analogs led to the identification of the tadalafil precursors, including GR30040X, as reflected in Scheme 5. During the trial, our lawyers were able to demonstrate that this description could not possibly be true because 1) the scientific paper in question was not published until after Glaxo conducted its initial tests on GR30040X, and 2) the Glaxo team did not conduct its initial tests on \( \beta \)-carboline analogs for at least another month after that. We were also able to demonstrate that the compounds that were tested by Glaxo on April 23, 1992, could have been identified by Glaxo through a substructure search of its chemical database performed using the basic skeleton of our IBMX analog. Unable to continue to rely upon their original \( \beta \)-carboline story, ICOS asserted that those compounds that were tested on April 23 were identified through a substructure search drawn from an altogether different compound, thus presenting the court with two competing substructure search theories (Scheme 6).

Although the trial court found that there was no evidence that the Glaxo scientists would have had any basis for choosing those three rings for a substructure search and that we had clearly made a contribution to Glaxo’s efforts, the trial court found the competing theories to be equally plausible. Because the burden of proof rested with Vanderbilt, the trial court found in favor of ICOS.

We appealed the decision, and the three-judge appellate court agreed unanimously that the trial court had applied an erroneous standard for determining whether we had contributed sufficiently to be named joint inventors. However, although one of the judges believed that error warranted further consideration by the trial court, the other two voted to affirm the decision in favor of ICOS.

Sometimes, I regret the loss of scientific output because of my nearly five-year involvement in the lawsuit proceed-
ings that came to naught for Vanderbilt, Sharron, Raja, and me, but it was an exceptional adventure outside the scientific realm. We are deeply grateful to Vanderbilt and our excellent team of lawyers for supporting us throughout the legal proceedings. Among several realizations, it seemed to me that, in patent cases, the courts tend to emphasize the importance of the invention of the final product, tadalafil in this case, whereas the scientific world tends to emphasize the importance of the basic discoveries leading up to the final product.

Concluding Remarks

As I now reflect on my career, I ask myself, “Would I do anything differently?” I probably would not, at least not concerning the major decisions of my scientific career. At times, there were disappointments, such as research grants denied, questionable choices of effort devoted to different biological questions, and the unsuccessful lawsuit, but the rewards were gratifying. I urge young people to adopt a scientific career. You will be appropriately challenged; you will meet many interesting people from diverse cultures; you will feel the ecstasy of discovery; and you will contribute to the improvement of the health and welfare of all living things. In these Reflections, I hope that I have conveyed the fact that solid basic science often leads to such improvements.

There are many more vocations for scientists to enter nowadays than during my era, and these areas have already been improved by scientific inputs. In addition to traditional fields, such as medicine, the fields of law, sports, police work, the military, and many others have embraced scientific approaches, such as demanding rigorous proof for claims that are made. The need for scientists in additional fields will increase as more people become convinced that the scientific method works.

I am semiretired now, but remain at Vanderbilt. I do very little research, but I sorely miss it. I miss my students and all of my scientific colleagues and staff (Fig. 9). I miss our lab discussions and our social gatherings. I miss our backpacking in the Smokies, canoe trips on the Caney Fork river, arrowhead hunting on the middle Tennessee riverbanks and plowed fields, and the camaraderie of our departmental softball, basketball, volleyball, and tennis teams. I admit continuation of some of these activities, and interactions with my granddaughter, Evee, have added much enjoyment in recent years (Fig. 10).

Acknowledgments—I am deeply grateful to the many scientists, including undergraduate students, medical students, graduate students, postdoctoral fellows, mentors, collaborators, and visiting scientists, with whom I have worked to achieve what has been described in this Reflections. All contributed to the eventual invention of PDE5 inhibitors for treatment of erectile dysfunction. Obviously, when I use the first person to describe a discovery, it is meant to include the other individuals who participated in that discovery. If they are not mentioned in the text, many, although not all of them, are listed in the references. I am also thankful to Sharron Francis, Tom Lincoln, Elaine Ritter, Roger Colbran, Bob Brennen, Sekhar Konjeti, Owen McGuinness, and Roger Cone for reading the manuscript and offering helpful suggestions.

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