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Sharron H. Francis
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Phosphodiesterases as Drug Targets

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Preface

The cyclic nucleotides, cAMP and cGMP perform ubiquitous signaling roles. In mammals, one or other or both are connected with the regulation of a panoply of key processes that include learning and memory, cell cycle control, differentiation, inflammation, cardiac functioning, smooth muscle relaxation/contraction, and visual signal transduction, to name but a few. As such, there has been much interest over the years in trying to identify, resolve, and comprehend the signaling systems associated with cAMP and cGMP in health and disease and to determine how this knowledge can be translated to generate novel means of therapeutic intervention.

Psychologically, most of us seem to be geared to a greater appreciation of the creation of objects and material rather than their destruction. Invariably, this translates into our collective approach to scientific problems. Certainly, in this regard, the G-protein-coupled receptor (GPCR)-stimulated generation of cAMP has attracted enormous attention over the past three decades. This interest has been translated into effective therapeutics that have exploited the diversity of receptor subtypes and their cell type-specific patterns of expression and been greatly facilitated as their binding site is exposed at the cell surface. More recently, enthusiasm for studying the enzymes that generate cGMP and mediate the cGMP-signaling pathway and its potential for drug targets have emerged. Efforts to target pharmacologically the enzymes that break down cyclic nucleotides, i.e., the cyclic nucleotide phosphodiesterases (PDEs) have more recently materialized, and some of the drugs produced in such programs have proven to be spectacularly successful in the clinic.

For many years, it has been an apparent conundrum that many organisms, including mammals together with lower organisms such as *Drosophila melanogaster* and *Caenhorhabidits elegans*, have stockpiled a mass of PDEs that catalyze the destruction of cAMP and cGMP. Work from many laboratories, including those contributing to this volume, has shed light on why nature has seen fit to not only conserve this diversity but also to elaborate on it throughout evolution. Thus, we see PDEs that have different affinities for cAMP and cGMP, such that low K_m enzymes can scavenge and ensure that signaling systems are truly switched off in resting cells, but then there are sets of PDEs with higher K_m values that "kick in" as cyclases are activated to produce more cAMP or cGMP in cells stimulated by various signals. PDEs also have regulatory features that (a) mediate negative

feedback pathways, which accelerate cyclic nucleotide hydrolysis; (b) provide for selective localization of particular PDE isoforms within a cell so as to confer precise regulatory control upon specific, spatially constrained cellular processes; and (c) provide for cross-talk with other signaling systems so as to integrate cellular responses. Some PDEs are activated through phosphorylation by cyclic nucleotide-regulated protein kinases and so provide a pivotal part of the cellular desensitization mechanism to the corresponding cyclic nucleotides.

Finally, over the last decade, we have seen the advent of genetically encoded sensors for both cAMP and cGMP. These have allowed for the visual appreciation of a phenomenon that has been inferred but, until this time, neither fully proven nor fully accepted, namely that both cAMP- and cGMP-signaling events are compartmentalized in cells. However, for this, you need the targeted, rather than the mass destruction of cyclic nucleotides, for such gradients to form in cells. Tethered subpopulations of cAMP and cGMP sensors subsequently interpret these PDE-shaped gradients. This new understanding offers a pivotal insight into the "why so many PDEs" conundrum. Thus, a large library of PDEs is available where individual isoforms are expressed on a cell type-specific basis. This allows targeting of particular PDEs to specific intracellular sites, membranes, and signaling complexes within cells so as to shape gradients and gate the activation of sensors around them. It is the diversity of PDEs, expressed on a cell type-specific basis with specific functional roles that offers potential for therapeutic exploitation.

The hope for the first PDE therapeutic was aimed at developing selective inhibitors of PDE3 for treatment of heart failure. The first clinical trials were performed with milrinone, which although enhancing cardiac function as hoped, was never mass-marketed as it gave rise to an increase in death rates due to arrhythmias. However, these unfortunate effects were most likely exacerbated by the fact that the patient cohort evaluated were end-stage patients; moreover, milrinone at higher concentrations can inhibit other PDEs. Nevertheless, milrinone is still used under hospital supervision and, furthermore, the highly specific and high affinity PDE3-selective inhibitor, cilostamide is approved for use in intermittent claudication and has no known arrhythmiogenic effect.

The concept that PDEs are promising drug targets has been spectacularly extended with selective inhibitors for the cGMP-hydrolysing PDE5. These compounds found a commercial niche for treating penile erectile dysfunction although the first of these compounds had ancestors that originated from programs designed to develop drugs for treatment of heart disease. Since then PDE5-selective inhibitors have progressed to being approved for treatment of pulmonary hypertension and, ironically, may progress back to a new found utility in the treatment of heart disease and other cardiovascular maladies. There has also been a huge effort by the pharmaceutical industry in developing selective inhibitors for members of the PDE4 family. Unfortunately in the race to do this, the generation of a multitude of such compounds ran well-ahead not only of our understanding of both the diversity of isoforms within the four genes PDE4 family, but also ahead of our understanding of their functional roles and structures. Undoubtedly, this has led to a lot of frustration over the years in appreciating which PDE4 isoform is the

"true target" in a particular tissue/cell type and how to deal with adverse side effects of such drugs, such as nausea. Nevertheless, we now have just seen approval for the first PDE4-selective inhibitor, which is being used as a therapeutic to treat chronic obstructive pulmonary disease (COPD). However, recent major advances in our understanding that particular PDE4 isoforms can perform specific functional roles through targeting to signaling complexes, plus new structural insights into how regulatory domains interact with catalytic units bodes well for subsequent generations of PDE4-selective inhibitors. These are likely to address additional therapeutic areas including cognition, psychosis, and cancer. In addition to this, a number of research programs are vigorously pursuing inhibitors of PDE10 for treatment of neuropsychological disorders.

This is then an exciting time for PDE research and development of drugs that target specific enzymes within the myriad of PDEs encoded by the human genome. Each PDE appears to have a specific functional role that affords novel opportunities for development of specific therapeutic interventions. The ability for genetic ablation of particular PDEs, coupled with siRNA-mediated knockdown of specific PDEs and the use of novel dominant negative approaches provide means of comprehending function and further defining potential targets. Furthermore, the huge increase in structural insight of catalytic and regulatory domains of PDEs has transformed our ability to optimize the design of specific inhibitors, and we look forward to the insights that will be derived from the resolution of more complex structures involving not only full-length PDEs, but also for PDEs in complex with specific partner proteins. The ability to assess changes in cAMP and cGMP around specific functional signaling modules will allow not only new biological insights but will also provide the potential for screening for new therapeutics.

Given the limitation in budget, we are inevitably constrained in what we can present. However, in the collection of articles in this volume, we hope to give you a taste of some of the exciting ideas and developments that are currently emerging in this dynamic and important field and how future therapeutic exploitation is currently shaping up. We hope that you enjoy and are inspired by reading them as much as we have been.

Glasgow, UK — Miles D. Houslay
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Phosphodiesterase Inhibitors: History of Pharmacology

Christian Schudt, Armin Hatzelmann, Rolf Beume, and Hermann Tenor

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Abstract The first pharmacological investigations of phosphodiesterase (PDE) inhibitors were developed with the clinical efficacies of drugs isolated from coffee, cacao and tea but only later their relevant ingredients were identified as xanthines that act as PDE. With its diuretic, inotropic and bronchodilating clinical efficacy, use of theophylline anticipated the clinical goals, which were later approached with the first-generation of weakly selective PDE inhibitors in the period from 1980 to 1990. Pharmacological and clinical research with these early compounds provided a vast pool of information regarding desired and adverse actions – although most of these new drugs had to be discontinued due to severe adverse effects. The pharmacological models for cardiac, vascular and respiratory indications were analysed for their PDE isoenzyme profiles, and when biochemical and molecular biological approaches expanded our knowledge of the PDE superfamily, the purified isoenzymes that were now available opened the door for more systematic studies of inhibitors and for generation of highly selective isoenzyme-specific drugs. The development of simple screening models and clinically relevant indication models reflecting the growing knowledge about pathomechanisms of disease are summarised here for today's successful application of highly selective PDE3, PDE4 and PDE5 inhibitors. The interplay of serendipitous discoveries, the establishment of intelligent pharmacological models and the knowledge gain by research results with new substances is reviewed. The broad efficacies of new substances in vitro, the enormous biodiversity of the PDE isoenzyme family and the sophisticated biochemical pharmacology enabled Viagra to be the first success story in the field of PDE inhibitor drug development, but probably more success stories will follow.

Keywords Asthma · COPD · PDE inhibitors · PDE4 inhibitors

1 Introduction

Drug discovery up to 1970 usually happened in small research departments which represented a minor activity of a chemical company. The research team consisted of chemists and pharmacologists. The latter were educated as medical doctors and thus knew the areas of urgent medical need. This team decided about the goals of research (not the board) – mainly on the basis of available resources – and defined

the three essentials (1) the disease indication, (2) the chemical compounds which should be examined or derivatised and (3) the armamentarium of models which were available and could be used for the intended project.

The clinical indications needed to be (1) painful, (2) life-threatening and (3) "ethical". The pharmacological models had to be designed to deliver read-outs for (1) therapeutical efficacy, (2) dosage and (3) side effects. These parameters were evaluated in anaesthetised intact animals suitable for measuring respiration and cardiovascular and metabolic parameters. Efficacy and potency of various compounds were compared and quantified in isolated organ preparations. These models detected either contractions or relaxations of muscle preparations that had been developed since the beginning of the twentieth century. They provided dose–response curves, were independent from the whole organism and contributed essentially to the selection of new chemical entities (NCEs), which for patent and commercial reasons needed to show "significant advantage and progress" over existing medications.

Most problematic was the source of chemicals used for investigations. The available compound sources were (1) antagonists (of or toward) hormones and mediators, (2) alkaloids which were highly effective but tremendously toxic and (3) some heterocyclic compounds which were used as diuretics, antihypertensives, cardiotonics, pain killers or anti-infectives. Each new project had to be initiated with a long and careful search in the chemical literature for identification of structures which might eventually be active in the biological system of question. The yield, however, was usually low, and even in the middle of 1970, most new developments were called "me-too" compounds developed on the basis of already existing, less effective drugs. After performing toxicologic testing, these substances could eventually be administered to humans. The important tasks of pharmacology were to (1) provide evidence for efficacy and proof of concept in vivo, (2) identify a relevant dose in combination with pharmacokinetics and (3) define a safe "first dose in man". Elucidation of the mechanism of action (MoA) was desirable but not considered to really be necessary. The area of phosphodiesterase (PDE) inhibitor provides several examples for this fast track path to clinical application in the period from 1977 to 1985 (summarised in Table 1).

In the years between 1950 and 1970, basic biochemical research was successful in isolating many enzymes and clarifying their functions, identifying key enzymes of metabolism and starting to analyse molecular mechanisms of diseases. Examples for pioneering research with prominent key enzymes, which were snatched up by pharmaceutical industrial laboratories, were HMG-CoA reductase for cholesterol synthesis, angiotensin-converting enzyme (ACE) for regulation of blood pressure, xanthine oxidase for gout and Na/K-ATPase and the H/K-ATPase for gastric acid secretion. These drug targets were established with considerable success in the years between 1970 and 1980. Enzymes and isolated cells – primary and immortalised – provided a completely new armamentarium for use in pharmacological research. About 10- to 100-fold more measurements per day became possible and identification of true new lead structures from the pool of stored chemicals in each company became a real possibility. Beginning in 1990, highly automated versions

Table 1 Clinical studies with prototypical PDE inhibitors

Inhibitor	PDE	Indication	Assessment	References
Theophylline	Unsel	Asthma, COPD	Symptoms	Hirsch (1922)
Amrinone	3	Chronic heart failure	Myocardial contractility	Benotti et al. (1978)
Enoximone	3	Pulmonary hypertension, COPD	PAH, bronchodilation	Leeman et al. (1987)
Rolipram	4	Depression	Depression scale	Horowski et al. (1985)
Ro 20-1724	4	Psoriasis	Disease score	Stawiski et al. (1979)
Denbufylline	4	Multi-infarct dementia	Psychometry	O'Connolly et al. (1988)
Zaprinast	5	Exercise-induced asthma	FEV1 during exercise	Rudd et al. (1983)
Zardaverine	3/4	Asthma	FEV1	Brunnée et al. (1992)
Papaverine	Unsel	Erectile dysfunction	Penile erection	Brindley (1982)
Benafentrine	3/4	Bronchoconstriction in volunteers	Methacholine-induced FEV1decrease	Foster et al. (1992)
Tolafentrine	3/4	Pulmonary hypertension	PAH	Ghofrani et al. (2002b)
Sildenafil	5	Erectile dysfunction	Penile erection	Boolell et al. (1996)
		Pulmonary hypertension	PAH, exercise tolerance	Ghofrani et al. (2002a)
Cilostazol	3	Intermittent claudication	Walking distance	Kumar et al. (2007)
Piclamilast	4	Rheumatoid arthritis	Disease score	Chikanza et al. (1996)
Cilomilast	4	COPD	FEV1	Compton et al. (2001)
Roflumilast	4	COPD	FEV1, acute exacerbations	Calverley et al. (2009)

Clinical studies are listed which have been performed with prototypical, advanced and "first-in class" PDE inhibitors. Theophylline and papaverine are denominated as unselective (unsel) PDE inhibitors

of these procedures were established, and high throughput screens testing more than 10,000 compounds per day were the pride of every scientific board. The role of classical pharmacology was now to compare compounds preselected by biochemical methods and to characterise candidates with regard to (1) in vivo potency, (2) duration of action, (3) efficacy and (4) adverse effects. In view of the change in the approach of drug development to specific (enzyme) targets, PDE research was a latecomer due to the continuous discovery process of multiple PDE activities and the insufficient insight into relationship between PDE subtypes and pathological functions. However, as the complexities of the PDE superfamily were slowly defined and isolated PDEs became available for study, a greater understanding of their function emerged.

A history of the pharmacology for drugs that inhibit PDE needs to start with the role of the ancient PDE inhibitor theophylline, which was in use therapeutically long before its biochemical action was characterised in 1958. Independently of its biochemical function, theophylline stimulated myriads of pharmacological investigations. Its clinical use followed the pharmacological studies, and despite being

accompanied by substantial adverse effects (AEs), its use anticipated efficacy in several diseases which are currently treated by selective PDE inhibitors or will be in the near future. After identification of PDEs and theophylline as a PDE inhibitor in 1958, it took nearly 20 years before the clinical potential of new PDE inhibitors was recognised. Thus, development of new PDE inhibitor drugs started at the time when the classical drug discovery process, which was an intimate interplay between pairs of laboratories from medicinal chemistry and pharmacology, was revolutionised by biochemists who introduced new methodologies employing purified enzymes, receptors or cells. The success of drug research programs depended on the understanding of PDEs, which appeared to be peculiarly complex due to the explosion from originally one enzyme in 1958 to over five isoenzymes in 1985, to a protein superfamily that is now known to contain more than 100 members. Since members of the PDE superfamily are expressed in most, if not all, tissues, each desired effect is potentially accompanied by other undesired side effects. In order to analyse and discriminate between desired and adverse effects, a broad understanding of PDE distribution needed to be established. In this historical overview, we concentrate on PDE3, PDE4 and PDE5, where certain inhibitors are approaching or have already reached the goal of approval for use as medications.

2 The Ancestor Theophylline: A Multitalent with Bad Character

2.1 Asthma and Caffeine

Therapeutic efficacy of caffeine in asthma patients was originally observed and meticulously described in 1860 by the American physician Henry Hyde Salter in his book “On asthma, its pathology and treatment” (Salter 1860; Persson 1985). Salter suffered from asthma himself and this intimate relationship to the disease resulted in precise observations and quite modern conclusions. He observed that bronchospasms may be induced either by exercise and cold air or by the emanations of domestic cats or hay. He thus characterised hyperreponsiveness as “excessive irritability” of the airways in a lung of “perfect organic health”. He discriminated these acutely occurring phases of dyspnea from more chronic airway disease, which occur as a consequence of bronchitis or of cardiac failure (“cardiac asthma”). He further realised that bronchospasms could be diminished by “sudden alarm, fright, surprise or pleasant excitement” (an effect that is replicated by ß-mimetics), smoking dried leaves of *Dattura stramonium* (which contains anti-cholinergics that mimic atropine) or by “two morning cups of strong coffee” (which contains a mix of xanthines corresponding to a dose of ~300 mg theophylline (May 1974). In view of today’s knowledge, it seems that Salter’s observations might have been sufficient for the development of various asthma and/or chronic obstructive pulmonary disorder (COPD) therapeutics as they exist today. However, systematic chemistry and pharmacological protocols to develop such drugs for medicinal uses had yet to be developed.

2.2 Xanthines for Medical Remedies

Three xanthines (caffeine, theobromine and theophylline) had been isolated from beans of coffee and cacao and from tea leaves in 1820, 1842 and 1888, respectively, but it was not until 1895 that the chemical structures were analysed and finally proven via chemical synthesis (Fischer and Ach 1895). The first pharmacological investigation using these compounds was performed in an animal model for urine production and was published in 1887 by the pharmacologist Schroeder (1887). He canulised the urether of anaesthetised rabbits and observed that after intravenous administration of theobromine, urine production increased nearly tenfold. This discovery was the birth of the xanthines as "diuretics". This observation was highly relevant, and its clinical application met an important medical need because it gave relief to patients with leg and lung edema (called "dropsy") caused by heart failure. This early pharmacological research success stimulated entrepreneurs in chemical industry to launch products containing xanthine mixtures such as "Diuretin" (Knoll is the company that sold that product from 1889), "Agurin" (Bayer1901) or "Theocin" (Byk Gulden 1902) (Rau 2001). One of the major drawbacks of theobromine and theophylline was their low solubility in water. A freshly employed chemist at Byk Gulden (Grueter 1910) solved the problem by creating a complex containing theophylline and ethylenediamine. This formulation resulted in the first preparation of theophylline for oral and parenteral application and was called "Euphylline". Heinrich Byk, an entrepreneur who had founded a chemical company in 1873, instantly decided to build a new pharmaceutical factory where Euphylline was produced in various forms, e.g., as an infusion, tablet and suppositories. Although this was a provocation for established pharmacists, who had the privilege and responsibility to mix medications with their own competence, this new drug conquered the market in Europe and USA and was prescribed for the treatment of angina, coronary sclerosis and so-called "hydrops". By around 1900, 16 of 10,000 Americans in USA received a prescription of one of the available popular xanthine medications. A historical summary is given in Table 2.

Table 2 Early historical milestones of PDE inhibitor research

1820	Isolation of caffeine, theobromine (1842) and theophylline (1888)
1860	H.H. Salter discovers coffee as effective treatment against asthma
1887	W.v. Schroeder determines diuretic function of theobromine in pharmacological experiments
1889	Mixtures of theophylline and theobromine are used as treatment for cardiac insufficiency
1895	O. Langendorff establishes isolated perfused heart
1899	K. Hedbom describes increased contractility of rabbit hearts after caffeine
1912	J. Pilcher finds inotropic function of xanthines
1912	P. Trendelenburg establishes isolated bronchi from the cow
1922	S. Hirsch compares clinical efficacy with pharmacological results and defines xanthines as bronchodilators
1936	G. Herrmann and P. Greene rediscover successful treatment of status asthmaticus with theophylline
1958	T. Rall and E. Sutherland discover PDEs and identify theophylline as PDE inhibitor

Discovery of xanthines, their pharmacological and clinical applications are listed

2.3 Rationale for Pharmacology

Driven by the widespread medical use of xanthines and their success in treatment of cardiac insufficiency, the influence of these compounds on cardiac functions were studied in the emerging pharmacological models of the time. Isolated, perfused heart preparations were established by Langendorff (1895), and the xanthine-induced increase of contractile power of the left ventricle was demonstrated using this model (Pilcher 1912; Plant 1914). Furthermore, in vitro isolated smooth muscle tissue preparations including blood vessels and segments of intestinal wall (Magnus 1904) were used to demonstrate the effect of xanthines to decrease contractile force and promote relaxation. These effects contributed a further rationale for the use of xanthines for treatment of cardiovascular diseases.

Around 1920, Euphylline was the mainstay for treatment of cardiovascular diseases, and it was Samson Hirsch, MD who shifted the focus to airway pathophysiology (Hirsch 1922). Using suppositories containing a mixture of theobromine and theophylline (ratio 1:2 and called "spasmopurin"), he treated patients experiencing bronchospasms from various origins. He discriminated between young patients with "endogenous asthma" from elderly patients with concomitant "bronchitis", "circulatory and cardiac insufficiency" and peripheral edema, which were called "cardiac and/or renal asthma". In both conditions, he observed that Euphylline produced fast relief of dyspnea and bronchospasms as well as a long-term amelioration of the general condition of the patient. He recommends the diuretin/spasmopurin treatment for both (1) "antispasmodic bronchodilatation" and also (2) for "prophylactic treatment" and thereby recognises that a chronic disease underlies the acute symptoms.

Being already familiar with the pharmacological experiments of Trendelenburg using isolated tracheal muscles (Trendelenburg 1912), Hirsch then reinforced his clinical observations by conducting in vitro experiments. He measured the influence of the xanthine mixture on isolated bovine bronchial muscle preparations. In the summary of his unique publication on the combined clinical and pharmacological investigations of bronchospasms, he states that (1) theobromine and theophylline are bronchodilators, and (2) bronchi are relaxed independent of their spasmogenic origin. Moreover, he recommends that both drugs should be used not only as "diuretics" but also as "bronchodilators". This was Hirsch's only published paper with pharmacological results, and although carefully described, these discoveries did not gain general acceptance or clinical application (May 1974). The reason that such an important discovery was not recognised seems to be twofold: (1) within the title of this paper the terms "asthma" and "theophylline" were not included and thus it may have missed public recognition; (2) apparently, there were no pharmacological societies or clinical opinion leaders available to spread these observations to those in clinical practice. Similar pharmacological results had been obtained by Macht and Ting but without accompanying clinical results (Macht and Ting 1921). It took another 15 years until the bronchospasmolytic potential of theophylline in the status asthmaticus was reinvented in clinical studies

independently by Herrmann and Greene (Hermann et al. 1937; Greene and Paul 1937). They showed that a very slow intravenous injection of 480 mg theophylline over 2–5 min resulted in a "prompt and persistent relief" from dyspnea. Based on the publication of these investigations, theophylline was promulgated as a bronchodilatory substance and became the mainstay of asthma therapy from 1940 onwards. In the years following 1980, theophylline treatment for asthma was increasingly displaced by inhalative glucocorticoids, but the anti-inflammatory activity of theophylline was still intensively investigated in the 1990s, and today further mechanisms for the action of low-dose theophylline, i.e., as a PI3K inhibitor and HDAC2 activator, are discussed (Barnes 2003a). Since, in addition to these effects, theophylline is also a potent antagonist of the anti-inflammatory A2-receptor-linked function of endogeneous adenosine, it seems that its characteristic therapeutic unreliability is based on the counteraction of its endogeneous anti- and pro-inflammatory mechanisms.

2.4 Narrow Therapeutic Window

Thus, in the first half of the twentieth century, theophylline had acquired a prominent position in the drug spectrum for use in counteracting various life-threatening disease states. It combined (1) inotropic, (2) diuretic and (3) bronchospasmolytic efficacies. However, this broad clinical spectrum was accompanied by severe, in some instances toxic side effects. Adverse events (AEs) on the cardiac, central nervous system and gastrointestinal functions such as (1) tachycardia, palpitations, tremor and arrhythmias, (2) headache and (3) nausea and vomiting were frequently experienced, but these problems were tolerated since the expectation in the tolerability vs. efficacy ratio in those days was low. Correct dosing was not possible because of the lack of knowledge of pharmacokinetics for these drugs, and the dose was determined by trial and error. We know today that proportionality of dose to blood concentration of theophylline is unusually weak (Ohta et al. 2004) and that even at low doses of 2 $\times$ 200 mg/day blood concentrations of $>$15 mg/l (in 13% of patients) followed by toxic AEs may occur. The most severe and frightening AEs of theophylline were seizures, which had already been published by Allard (1904). He describes two patients who were repeatedly injected with a relatively small dose of 300 mg theobromine. They had been rescued from dyspnea, increased their cardiac contractility, and their general condition had been significantly ameliorated. In spite of this considerable beneficial effect of the medication, these two patients suddenly and surprisingly were attacked by "epileptic convulsions" and died a few minutes later. This alarming finding was instantly confirmed in toxicological experiments with dogs and rabbits. Similar cramp phenomena were observed after diuretin administration when dogs died and a "lethal dose" of 500 mg/kg was evaluated. This dose was far greater than the critical doses in man (500 mg per patient), and a clear correlation from dog to man could not be drawn. Today, it is well known that blood concentrations established after a constant dose of

theophylline show massive deviations among individuals. The Ohta study which included nearly 3,800 patients underlined this unusually shaky ratio, and no significant relation between dose and AEs could be found (Ohta et al. 2004). Consequently, in clinical studies patients had to be preselected for AEs, and for the practitioner, theophylline had to be individually dosed after measuring blood concentrations (Wilkens et al. 1984) and even then the probability of AEs was high.

2.5 Theophylline as Tool for PDE Research

The identification of theophylline as a PDE inhibitor emerged in the initial experiments of Sutherland and Rall in 1957 when they opened the world of cAMP signalling (Rall and Sutherland 1958). They investigated hormone action on glycogenolysis in broken cell preparations and found that caffeine inhibited the basal, non-activated form of glycogen phosphorylase. They reasoned that addition of caffeine to the adrenaline-stimulated interconversion test might improve the detection of the activated phosphorylase. In contrast to their expectation, they observed a synergy of caffeine with adrenaline or glucagon in activating phosphorylase. From this result, they consequently hypothesised that caffeine might inhibit either the activity of the agent that destroyed their "heat-stable factor" or the phosphorylase-phosphatase (Butcher 1984). Later, when this heat-stable factor was identified as cAMP – the "golden bullet" for second messenger signalling – the enzymatic activity for breakdown was determined to be "phosphodiesterase activity" and concomitantly, caffeine and theophylline became the first recognised PDE inhibitors (Rall and Sutherland 1958). In the following years, the potentiation of adrenaline effects was shown in pharmacological and biochemical assay systems: inotropic responses in isolated perfused hearts (Rall and West 1963) and likewise in other tissues.

3 Pharmacological Models Need to Be Analysed Biochemically

3.1 Different Inhibitory Profile Indicate Multiple Enzymes

Papaverine was isolated in 1848 and introduced as the second PDE inhibitor after the xanthines. Pharmacological activity of both types of compounds were compared in a variety of contractile preparations including heart, vascular and intestinal smooth muscle, isolated bronchi and also in metabolic functions such as lipolysis of isolated fat cells or glycogenolysis in liver tissue. Papaverine functioned as an efficacious smooth muscle relaxant and was denominated a "direct vasodilator", but it showed comparably less inotropic activity in the heart or enhancement of lipolysis or glycogenolysis. Theophylline was weaker in potency and in vascular and intestinal muscular preparations where it predominantly potentiated adrenaline

effects, but it was equally effective in each system studied. These early observations of the different pharmacological and biochemical profiles of the PDE inhibitors was observed by several authors (e.g. Poech and Kukovetz 1971) and gave rise to the view that there must be multiple PDE enzymes and that different tissues may contain different PDEs.

3.2 Column Chromatography Profiles of PDEs and Development of the First Generation of PDE Inhibitors

Up to 1985, a variety of pharmacological models for PDE inhibitor research had been established, and the prominent ones are listed in Table 3. In order to understand the interference of the available old and new substances, a biochemical analysis clarifying PDE content and diversity of all these models appeared to be inevitable. Thompson and Appleman provided key pioneering experiments by successfully using anion-exchange chromatography for separation of a number of

Table 3 Isolated organs and tissues available in 1985 for investigation of functions of PDEs 3, 4, 5

Preparation	Pretreatment	Response	PDE
Aorta, rat	L-Phe	Relaxation	
Intact			PDE5 > PDE4
Denuded			PDE3 > PDE5
Pulmonary artery, rat	L-Phe	Relaxation	
Intact			PDE5
Denuded			PDE5
Coronary artery, gp	L-Phe	Relaxation	
Intact			PDE5
Denuded			PDE5
Perfused heart, gp	Spontaneous	Contractility dp/dt	PDE3
		Coronary flow	PDE5 > PDE3
		Heart rate	PDE3
		LV pressure	PDE3
Left atrium, gp	Electrical stimulation	Force of contraction	PDE3
Tracheal rings, gp			
Untreated	Spontaneous		PDE3, 4, 3/4
Sensitised	OVA challenge		PDE4
Lung strips, gp	Histamine, carbachol	Relaxation	PDE3

Isolated organ and tissue preparations have been developed from the end of the nineteenth century. Functional analysis was based on isometric force transduction for either contraction or relaxation. The preferred species for each preparation is mentioned (gp = guinea pig) and the pre-treatment to reach a contracted state ready for relaxation by PDE inhibitors is given

OVA ovalbumin, *L-Phe* L-phenylephrine

PDE isoenzymes (Thompson and Appleman 1971). Thompson and his associates separated PDEs from cardiac and cerebellum tissues (Thompson et al. 1979), and Hidaka and Polson resolved those of platelets and canine trachea (Hidaka and Asano 1976; Polson et al. 1982). Platelets and cardiac tissue each revealed three peaks of PDE activity, whereas in canine trachea five different peaks of PDE activity were resolved. The PDEs in each of the peaks were characterised with enzymologic criteria such as (1) substrate specificity (cAMP/cGMP), (2) substrate affinity and (3) calmodulin and cGMP activation, but the data were hardly comparable and, even worse, every author used his own nomenclature. The discrimination of five different PDE classes emerged only after the separation methods were more refined and more selective tools (activators/inhibitors) were applied for characterisation of the peaks. Much of the confusion concerning the PDEs was largely ended, and each PDE peak of tissue-specific elution pattern could be attributed to this system. However, other complications such as proteolysis and expression of myriads of alternative splice variants in some families continued to complicate understanding of these enzymes. The publication of Reeves in 1987 (Reeves et al. 1987b) clarified that the cardiac peak III can be further separated into two cAMP-hydrolyzing PDEs where the earlier eluting peak is the highly cAMP specific rolipram-sensitive (now known as PDE4) and the later eluting cGMP-inhibited cAMP-PDE (now known as PDE3). This publication marks the time point when the system of PDE1–PDE5 with their typical enzymological characteristics became established in most laboratories that were engaged in PDE research (Weishaar et al. 1985; Nicholson et al. 1989; Schudt et al. 1991a, b, c; Torphy and Cieslinsky 1989). The elegant concept of a protein superfamily of six PDE families with each family containing several members was composed by Joe Beavo (1988), and many later publications along with the contributions of other pioneers in the field (Marco Conti and Rick Heaslip) unfolded the whole world of >60 PDEs in 11 families (Beavo et al. 1994; Conti and Beavo 2007). The enormous biodiversity of these key regulatory enzymes and the view of their distribution in different tissues underlined the rationale for searching for new drugs with defined selectivity for one or more PDE families or subtypes.

Around 1985, a pool of around 30 PDE inhibitors with weak potencies and selectivities was available; these have been listed and their chemistry has been described extensively in excellent reviews of that time (Weishaar et al. 1985; Torphy and Undem 1991; Nicholson et al. 1991; Beavo 1988). Prominent representatives and important tools for research progress at that time were SKF 94120, SKF 94 836, milrinone and motapizone for PDE3, rolipram and Ro 20-1724 for PDE4 and zaprinast for PDE5. Milrinone, rolipram and zaprinast had already been studied in patients as cardiotonics, antidepressants and bronchodilators, respectively (see Table 1). Due to insufficient safety or low therapeutic efficacy, these developments had to be discontinued. The positive aspects of these early and engaged trials was the demonstration that therapeutic efficacy in principle is possible and can be improved. Further, the recognition of AEs and of the necessity to study and understand their biochemical mechanisms was of considerable value for future research (for details of rolipram studies, see following chapters). On the