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Review

Calmodulin-dependent cyclic nucleotide phosphodiesterase (PDE1)

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Abstract. Ca^{2+}/calmodulin-dependent cyclic nucleotide phosphodiesterase (PDE1) is one of the key enzymes involved in the complex interactions between the cyclic nucleotide and Ca^{2+} second messenger systems. Currently, three genes encode PDE1, and alternate splicing of these genes gives rise to functionally different isozymes which exhibit distinct catalytic and regulatory properties. Some isozymes have similar kinetic and immunological properties but are differentially regulated by Ca^{2+} and calmodulin. These isozymes also differ in their mechanism of regulation by phosphorylation. Analysis of various regulatory reactions involving Ca^{2+} and cyclic adenosine monophosphate (cAMP) has revealed the importance of the time dependence of these reactions during cell activation; however, no measurement is available for the time of occurrence of specific regulatory reactions. cAMP-signalling systems provide a pivotal centre for achieving crosstalk regulation by various signalling pathways. It has been proposed that polypeptide sequences enriched in proline (P), glutamate (E), serine (S) and threonine (T), known as PEST motifs, serve as putative intramolecular signals for rapid proteolytic degradation by calpains. Calpains are Ca^{2+}-dependent cysteine proteases that regulate various enzymes, transcription factors and structural proteins through limited proteolysis. Isozyme PDE1A2 has a PEST motif and acts as a substrate for m-calpain. In this paper, we have described PDE1A2 regulation by calpains and its physiological implications. cAMP is an important component of the signal transduction pathway and plays an integral role in various physiological processes such as gene transcription, various neuronal functions, cardiac muscle contraction, vascular relaxation, cell proliferation and a host of other functions. It is important to identify the cellular processes where PDE isoform(s) and cAMP response are altered. This will lead to better understanding of the pathology of disease states and development of novel therapeutics. The different PDE1 isozymes, although similar in kinetic properties, can be distinguished by various pharmacological agents. Our recent understanding of the role of PDE1 inhibitors such as ginseng, dihydropyridine antagonists and antiparkinsonian agents are described in this review. The exact function of PDE1 isozymes in various pathophysiological processes is not clear because most of the studies have been carried out in vitro; therefore, it is essential that further research be directed to in vivo studies.

Key words. Calcium; calmodulin; phosphodiesterase; protein kinase; inhibitors; calpains.

Introduction

The purpose of this review article is to summarize some of the most significant advances related to PDE1 that

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have been carried out in our laboratory. Reviews on this subject have been published earlier [1–6]. We have tried our best to compile all recent significant work on PDE1 rather than providing an encyclopedia on all earlier published work. However, we have made an attempt to highlight earlier important findings which have been a foundation in the field of PDE1.

In general, intracellular second messengers such as cAMP and cyclic guanosine monophosphate (cGMP), undergo rapid concentration changes in response to a wide variety of cell-specific stimuli. To a large extent, the concentrations of these second messengers are determined by the relative synthetic activity of adenylate cyclase and degradative activity of cyclic nucleotide phosphodiesterase (PDE). Cyclic nucleotide hydrolysis activity was first demonstrated by Sutherland and Rall [7] after their discovery of cAMP. Hydrolysis of the cyclic nucleotides by PDE is the mechanism for their degradation. Despite their ubiquitous occurrence in biological systems, the cyclic nucleotides are not known to be substrates for any other enzymatic reactions. In most tissues, PDE exists in multiple forms which can be separated by conventional protein fractionation procedures and have been shown to exhibit distinct catalytic, regulatory and immunological properties. The multiple forms of PDE depend on the tissues and/or the protein isolation techniques: some forms may be biochemical artifacts due to partial proteolysis or binding to other cellular components (for reviews see [8–10]). Initially, the multiple forms were classified into three categories: cAMP PDE, cGMP PDE and PDEs based on their marked differences in relative affinities towards cyclic nucleotides [8, 9]. On the other hand, Beavo et al. [10] grouped these forms into various major categories such as Ca^{2+}/calmodulin-stimulated PDE, cGMP-stimulated PDE, cGMP-inhibited PDE and cAMP-stimulated PDE, which were based on regulatory and kinetic properties. Since the list of new PDE isozymes was rapidly expanding, a new nomenclature was developed at the American Society for Pharmacology and Experimental Therapeutics Colloquium on Multiple Phosphodiesterases [11]. This nomenclature of the multiple isozymes has been derived based on the primary structures of different PDEs. It is now known that at least three different genes encode the Ca^{2+}/CaM-stimulated PDE family; they are named PDE1A, PDE1B and PDE1C. The complementary DNAs (cDNAs) encoding the brain 63-kDa PDE1 isozyme (PDE1B1), brain 60-kDa PDE1 isozyme (PDE1A2), heart PDE1 (PDE1A1) and 70-kDa PDE1 (PDE1C) were suggested to represent subfamily members [11]. Recently new splice variants of mouse and human PDEs (PDE1C1, PDE1C2, PDE1C3, PDE1C4, PDE1C5, PDE1A3 and PDE1B1) have been reported [12–14]. They have been characterized in terms of their regulation by Ca^{2+}, sensitivity to inhibitors and tissue/cell-specific expression [12–14]. The genes for different PDEs undergo tissue-specific alternative splicing that generates structurally and functionally diverse gene products.

Discovery of PDE1

Kakiuchi and Yamazaki [15] originally demonstrated the existence of Ca^{2+}-stimulated PDE in rat brain. In addition, they discovered an endogenous protein factor in brain that could enhance the Ca^{2+} sensitivity of the enzyme [16]. It was established subsequently [17] that the protein factor was identical to the protein activator of PDE [later called calmodulin (CaM)] which was originally discovered by Cheung [18, 19]. Later, it was established that stimulation of the PDE required a physiological concentration of Ca^{2+} and CaM. Wang et al. [20] proposed a general mechanism for the activation of PDE by CaM, as represented by Scheme 1:

$$Ca^{2+} + CaM \rightleftharpoons Ca^{2+}\bullet CaM^{*}$$

$$Ca^{2+}\bullet CaM^{*} + PDE1 \rightleftharpoons Ca^{2+}\bullet CaM^{*}\bullet PDE1 \rightleftharpoons Ca^{2+}\bullet CaM^{*}\bullet PDE1^{*}$$

where CaM and PDE1 stand for calmodulin and calmodulin-dependent cyclic nucleotide phosphodiesterase respectively, and the superscript * denotes the activated state for the proteins.

The activity of PDE1 was found to be widely distributed in mammalian tissues and other eukaryotes [1–6]. PDE1 is one of the most intensively studied and best characterized of the multiple PDEs. The enzyme has been purified to homogenity and is well characterized in terms of its molecular properties [21–29]. Some of the physiochemical properties of the purified bovine brain PDE1 are summarized in table 1. All pure PDE1s essentially contain a single band on SDS-polyacry

Table 1. Physical parameters of PDE1.

Free PDE1	
sedimentation constant (S)	6.85
stoke radius (Å)	44.2
partial specific volume V, (ml/g)	0.726
molecular weight	
from sedimentation equilibrium	115,800
from sedimentation constant and Stokes radius	124,000
subunit weight	60,000
isoelectric point	4.85
absorbance (A278, 1%)	9.6
PDE1-calmodulin complex	
sedimentation constant (S)	8.0
stokes radius (Å)	48.0
molecular weight	159,000

For details of experimental conditions see Sharma et al. [22].

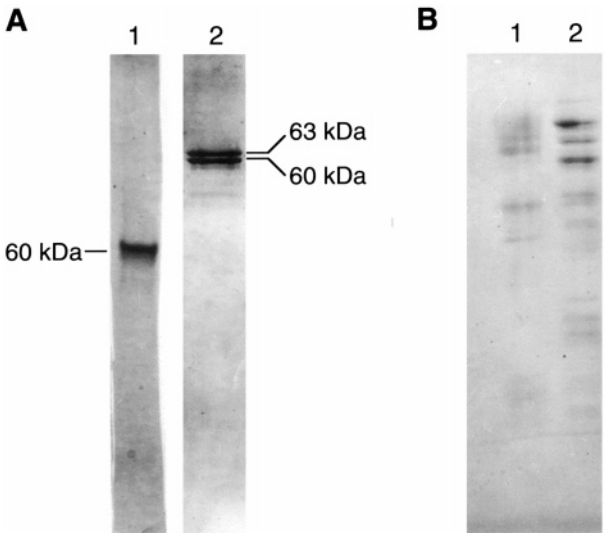

Figure 1. (*A*) SDS-PAGE of PDE1. Purified PDE1 was analyzed according to the methods of Weber and Osborn [32], lane 1, and Laemmli [33], lane 2. The polypeptides 63 and 60 kDa represent molecular masses. (*B*) Peptide maps of PDE1 isozymes: 63 kDa, lane 1, and 60 kDa, lane 2. For details of experimental conditions see Sharma et al. [34].

lamide gel electrophoresis (PAGE) and reported subunit molecular masses fall within a narrow range of 58–63 kDa [21–31]. Initially, variations in the subunit molecular masses were thought to arise from different purification procedures, SDS-PAGE conditions and/or from partial proteolysis. However, no significant differences in the enzymatic and molecular properties were noted from different tissues. It was suggested PDE1 consists of a single species. The availability of monoclonal antibodies has greatly helped to resolve these discrepencies.

Discovery of PDE1 isozymes

In an early study, Sharma et al. [22] demonstrated the purification of bovine brain PDE1 to apparent homogeneity on SDS-PAGE analysis according to the procedure of Weber and Osborn [32]. The purified protein revealed a single major band with a mobility corresponding to a molecular mass of 60 kDa (fig. 1A, lane 1). However, when the same preparation was analyzed using the procedure of Laemmli [33], two major polypeptides of approximately equal intensity were observed on SDS-PAGE (fig. 1A, lane 2). The molecular mass of these two polypeptides was observed to be 63 and 60 kDa (fig. 1A, lane 2), and the peptide maps of these polypeptides were also distinctly different (fig. 1B) [34]. When the immunocomplex of the PDE1 with C1 monoclonal antibody was subjected to sucrose density gradient centrifugation, it suggested that bovine brain contained three isozymes of PDE1 (fig. 2): two homodimeric proteins containing two subunits each of 63 and 60 kDa, respectively, and one heterodimeric protein form containing a 63- and 60-kDa subunit, respectively [34]. Therefore, we have used C1 monoclonal antibody immunoaffinity chromatography to resolve the purified bovine brain PDE1 into two fractions: one containing only a 63-kDa subunit and the other enriched with the 60-kDa subunit (fig. 3). These results suggest that the two prominent homodimeric isozymes, PDE1B1 and PDE1A2, are the predominant forms; however, PDE1A2 isozyme was always contaminated with 5–7% of heterodimer containing a PDE1B1 and PDE1A2 isozyme [34]. Since the brain is a highly heterogenous organ, containing many different cell types, it is possible that both major isozymes (PDE1B1 and PDE1A2) may have originated from different cell types. Over the years it has become clear that PDE1 exists as tissue specific isozymes [1–5].

The availability of monoclonal antibodies has greatly enhanced our knowledge in identification and purification of various PDE1 isozymes. Originally, the subunit molecular masses reported for purified bovine heart PDE1A1 were lower than bovine brain PDE1A2 [21, 27, 28, 34]. Hansen and Beavo [27] used an immunoaffinity chromatography on a PDE1A2-specific monoclonal antibody column to purify PDE1A2 and PDE1A1 from both bovine brain and bovine heart to homogeneity and further demonstrated that the bovine heart PDE1A1 had a lower subunit molecular mass (59 kDa) than did the bovine brain PDE1A2 [27]. Furthermore, a bovine PDE1A1 monoclonal antibody, ACAP-1, produced by Hansen and Beavo [35], was also found to cross-react with PDE1A2 but not PDE1B1 from bovine brain. Later, bovine heart PDE1A1 was purified in a single step from the total CaM-binding protein fraction using a C1 monoclonal antibody that reacts with PDE1A2 [28]. In addition, bovine lung-specific PDE1 isozyme was identified and purified by using C1 immunoaffinity gel [31]. In contrast to PDE1 from other tissues, the bovine lung PDE1 existed as a stable PDE1·CaM complex, similar to phosphorylase kinase which contains CaM as a subunit [36]. Sonnenburg et al. [37] reported that the lung PDE1 isozyme discovered by Sharma and Wang [31] (discussed later in this review) was identical to the brain PDE1A2 except for the amino-terminal 18 residues. This suggests that these isozymes are the alternate spliced products of the same gene. Recently, bovine eye PDE1 isozyme was identified and purified in our laboratory [29]. The eye PDE1 isozyme is immunologically similar to the bovine brain PDE1A2 and heart PDE1A1. Another PDE1 isozyme has been purified from bovine brain which has a molec-

ular mass of 150 kDa with a subunit molecular mass of 74 kDa. In the presence of Ca^{2+}-CaM, this novel isozyme shows increased affinity for cGMP and reduced affinity for cAMP [25]. It has also been reported that bovine testis contains a PDE1 isozyme with a molecular mass of 70 kDa, which dimerizes in the presence of Ca^{2+} and CaM to form a molecular mass of 180 kDa [38].

Kinetic properties

The kinetic properties of PDE1 have been examined by various laboratories from a variety of tissues and animal species (for reviews see [1, 4]). There were considerable discrepencies in kinetic properties because earlier studies were carried out with either crude extracts or partially purified samples. Originally, Kakiuchi et al. [39] demonstrated that the brain PDE1 had a higher affinity for cGMP than for cAMP. In order to elucidate the physiological significance of the PDE1 isozymes, the purified or highly purified preparations of PDE1 isozymes were used to determine the K_m and/or V_{max}. In general, PDE1 isozymes have a higher affinity towards cGMP than towards cAMP. Table 2 shows the kinetic properties of various PDE1 isozymes [25, 35, 40–47].

Differential activation by calmodulin and Ca^{2+}

Bovine brain PDE1 has been used in excellent detailed studies for the activation of PDE1 by CaM [48]. These studies were reviewed earlier [4, 5]. Although brain PDE1A2, heart PDE1A1, lung and eye PDE1 isozymes are almost identical in terms of immunological properties, they are differentially activated by CaM [28, 31, 35, 40, 49]. It may be possible that differential affinity for CaM may reflect subtle differences in Ca^{2+} activation of these PDE1 isozymes. The differences in CaM affinity exhibited by these enzymes may be related to the relative concentration of CaM in these tissues. It has been suggested that the differential CaM affinity is an

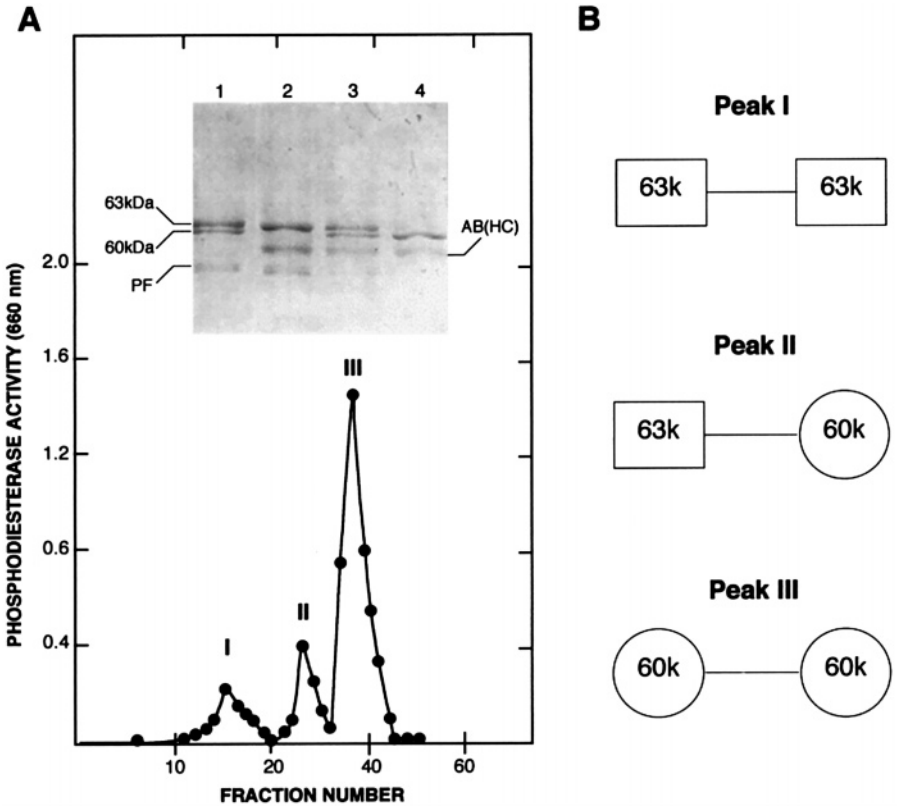

Figure 2. (*A*) Sucrose density gradient centrifugation profile of immunocomplexes of bovine brain PDE1 isozymes with C1 monoclonal antibody. Inset A: Sample for SDS-PAGE from peak I, II and III from PDE1 activity profile. Lane 1, PDE1 alone; lanes 2–4, peaks I, II and III from PDE1 activity profile. 63- and 60-kDa polypeptide from PDE1; and PF, proteolytic fragment from PDE1; AB (HC), antibody (heavy chain). (*B*) Schematic illustration of peaks I, II and III from the sucrose density gradient centrifugation.

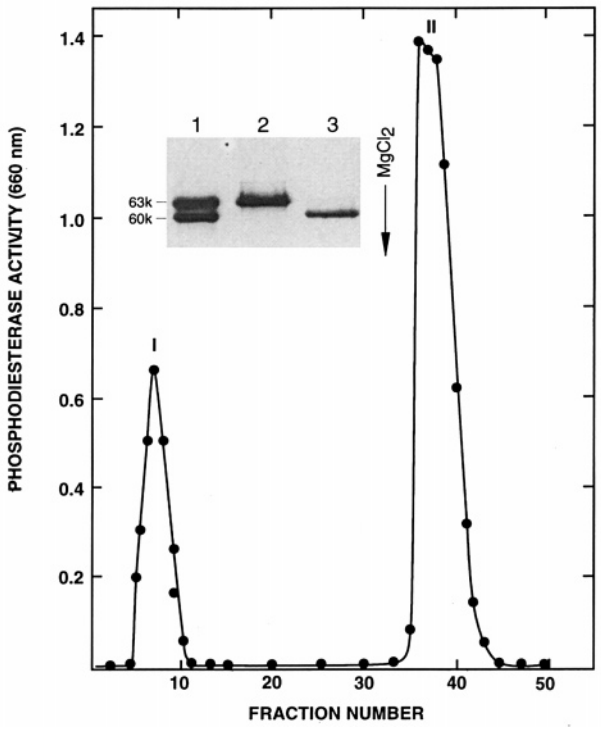

Figure 3. Separation of PDE1 isoenzymes by C1 antibody-Sepharose 4B column chromatography. For details of experimental conditions, see Sharma et al. [34]. Inset: SDS-gel electrophoresis analysis of original sample of PDE1, lane 1; pooled fractions of peak I, lane 2; pooled fractions of peak II, lane 3.

important mechanism by which the regulatory action of CaM may be fine-tuned [28, 35, 40]. However, the physiological significance of differential CaM affinity requires further research in this area. It is noteworthy that CaM concentration in mammalian brain is approximately 10 times higher than mammalian heart [50]. The PDE1A1 and eye PDE1 isozymes have a higher affinity for CaM than brain PDE1A2 [29]. Similarly, the pig brain PDE1 has been shown to have a lower affinity for CaM than the isozymes from pig artery [45]. In addition, Ca^{2+} and CaM interact synergistically in activation of PDE1 isozymes [40, 48]. When the CaM concentration is increased, the Ca^{2+} concentration required for half-maximal activation is decreased (table 3). Such synergistic interactions have been repeatedly shown for various CaM-dependent enzymes [51]. Although the physiological significance of the observed differential Ca^{2+} sensitivity of the PDE1 isozymes is not known, these studies suggest that the differential Ca^{2+} affinity of the tissue-specific isozymes may be a mechanism by which CaM regulatory reactions are adapted in the respective tissues.

Since bovine lung PDE1 isozyme contains CaM as a subunit, its activation by Ca^{2+} and CaM cannot be supported by the general mechanism [20]. As shown in figure 4, a change in CaM concentration had no effect on the Ca^{2+} concentration of the lung enzyme, suggesting that this isozyme does not undergo a Ca^{2+}-dependent reversible association with CaM. Furthermore, lung isozyme is not inhibited by CaM antagonists such as fluphenazine, compound 48/80 or by other CaM-binding proteins such as CaM-dependent phosphatase [31]. At present, the significance of CaM as a subunit is not known; however, the study suggested that the tightly bound CaM in lung isozyme is not subject to competition by other CaM-binding proteins after the increase in intracellular free Ca^{2+} concentration that occurs upon stimulation of the cell [31].

Interactions of the cAMP and Ca^{2+} second messenger systems

The intracellular second messenger Ca^{2+} is an important mediator in the regulation of a variety of biological processes [52–54]. The Ca^{2+} regulatory cascade is mediated by CaM [50]. In the case of the cAMP second messenger system, cAMP-dependent protein kinase(s) is the only established target protein in eukaryotic cells [55, 56]. It has become apparent that in many biological systems, the two second messengers, Ca^{2+} and cAMP, act in a concerted fashion to control regulatory process (fig. 5) [57]. Four regulatory pathways for the interactions between cAMP and Ca^{2+} second messenger systems have been observed. First, one second messenger regulates the metabolism of the other second messenger; for example, PDE1 controls cAMP concentration, and the phospholamban/Ca^{2+}-ATPase system regulates cytoplasmic Ca^{2+} concentration. Second, CaM-dependent enzymes are substrates of cAMP-dependent protein kinase, for example phosphorylase kinase and smooth muscle myosin light chain kinase. A third mechanism of cAMP and Ca^{2+}-CaM signal interaction involves regulation of glycogen synthase by both cAMP-dependent protein kinase and phosphorylase kinase. A fourth mode of interaction between the cAMP and Ca^{2+} signal systems involves the dephosphorylation of protein phosphatase inhibitor-1. Upon phosphorylation, protein phosphatase inhibitor-1 is active and inhibits the activity of protein phosphatase-1. When it is dephosphorylated by CaM-dependent protein phosphatase (calcineurin), protein phosphatase inhibitor-1 is inactivated, allowing protein phosphatase-1 to be activated.

Table 2. Kinetic properties of PDE1 isozymes from mammalian tissues.

Source of enzyme	K_m (μM)		V_{max} ratio (cAMP/cGMP)	Reference
	cAMP	cGMP		
Bovine brain				
60-kDa isoenzyme	12.0	1.2	0.3	40
63-kDa isoenzyme	35.0	2.7	1.8	
Bovine brain	29.0	2.7	0.07	25
Bovine spermatozoa	7.5, 95	–	–	41
Bovine coronary artery	2.5, 140	3.0, 17	0.18	42
Bovine lung	42.0	2.8	1.7	40
Bovine heart	215	9.0	3.3	43
	40.0	3.2	3.0	40
Guinea-pig left ventricle	0.8	0.9, 53	1.1	44
Human cardiac ventricle	0.75	1.0	1.0	44
Pig coronary artery	70.0	3.0	0.1	45
Rat brain				35
low molecular weight isoenzyme	–	–	1.2	
high molecular weight isoenzyme	–	–	0.68	
Rat pancreas				
peak I	0.35	0.16	1.4	46
peak II	9.0	1.7	1.1	
Rat testis				
peak I	1.0	1.0	–	47
peak II	30.0	3.0	–	
peak III	–	1.5	–	

Table reproduced from Wang et al. [4].

Regulation of PDE1 isozymes by phosphorylation

The major difference in the catalytic properties of the two bovine brain PDE1 isozymes appears to be their response to protein phosphorylation. The phosphorylation of PDE1 by cAMP-dependent protein kinase [22] and CaM-dependent protein kinase II [58] was reported prior to the discovery of the PDE1 isozymes [34]. Further studies were carried out using the purified bovine brain PDE1 isozymes and suggested that these isozymes are differentially regulated by second messenger-dependent phosphorylation reactions. The main difference is that the brain PDE1A2 and heart PDE1A1 are substrates of cAMP-dependent protein kinase, and phosphorylation is inhibited by Ca^{2+} and CaM [28, 59, 60], whereas brain PDE1B1 is phosphorylated by CaM-dependent protein kinase II in a Ca^{2+}/CaM-dependent manner [61–63]. The phosphorylation of both isozymes is accompanied by a decrease in the isozymes' affinity towards CaM and an accompanying increase in the Ca^{2+} concentrations required for the isozyme activation by CaM [28, 59–61, 63, 64]. PDE1A2 and PDE1B1 are phosphorylated by different protein kinases; however, both can be dephosphorylated by CaM-dependent protein phosphatase [59, 61]. This dephosphorylation is accompanied by an increase in the affinity of the

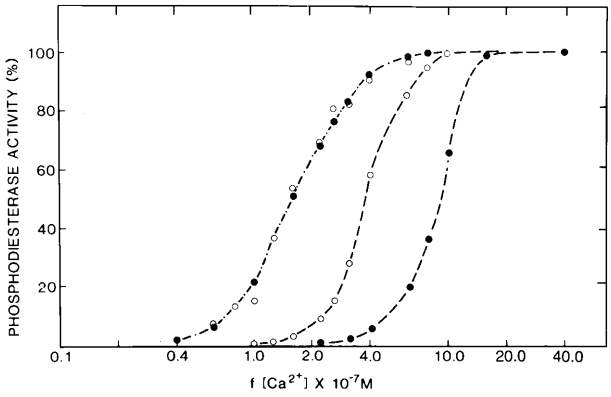

Figure 4. Effect of CaM on Ca^{2+}-dependence activation of lung PDE1 isozyme. CaM concentrations were 1 μM (●) and 10 μM (○) with bovine brain PDE1A2 isoenzyme (----) and lung PDE1 isoenzyme (-·-·-·-).

Table 3. Ca^{2+} activation of PDE1 isoenzymes at various concentrations of calmodulin.

[Calmodulin] (μM)	[Ca^{2+}] (μM) required for half-maximal activation		
	heart PDE1A1	brain PDE1A2	brain PDE1B1
0.04	0.50	nd	nd
1.0	0.08	0.90	0.70
10.0	0.01	0.35	0.30

For details see [40]. nd, not determined.

isozymes for CaM [59, 61]. Various second messenger effects on the PDE1 isozymes are summarized in table 4.

Role of PDE1A2 in the regulation of cAMP concentration

A working hypothesis describing the role of PDE1A2 in the coupling between the two messenger fluxes is presented in figure 6A. In most cases, cell activation involves transitory increase in both cAMP and cell Ca^{2+}. The operation of the different regulatory mechanisms on PDE1 may be temporally separated during the signal fluxes. The temporal separation of the regulatory reactions is a result of the Ca^{2+} and cAMP signal fluxes on the one hand and contributes to determining the intensity and duration of the fluxes on the other hand. Thus, an initial increase in cAMP concentration during cell activation may bring about phosphorylation of PDE1A2 and thereby prevent the enzyme from being activated by the low concentrations of Ca^{2+} existing at the early stages of cell activation. The hydrolysis of cAMP, therefore, would be inhibited coordinately with the stimulation of adenylate cyclase by external signals. This would ensure a rapid and sharp rise in intracellular cAMP. At later stages of cell activation, when intracellular free Ca^{2+} concentration is increased, the phosphatase reaction may be activated to reverse phosphorylation of the PDE1A2. The PDE1A2 then becomes fully activated by Ca^{2+} and CaM. Since Ca^{2+}-CaM can block PDE1A2 phosphorylation, the dephosphorylated state of the enzyme will be maintained even though the cAMP concentration may still be high in the cell. The concerted actions of these regulatory mechanisms on PDE1A2, namely the phosphatase reaction, Ca^{2+}-CaM stimulation of PDE1A2 and Ca^{2+}-CaM inhibition of phosphorylation, may bring about a rapid decline in cAMP concentration. A similar hypothesis has been proposed for heart PDE1A1 [57, 65].

Role of PDE1B1 in the regulation of cAMP concentration

All multiple regulatory actions of the PDE1B1 isozyme are dependent on Ca^{2+} and CaM (fig. 6B) [61, 63], whereas phosphorylation of PDE1A2 by cAMP-dependent protein kinase is inhibited by Ca^{2+} and CaM [59].

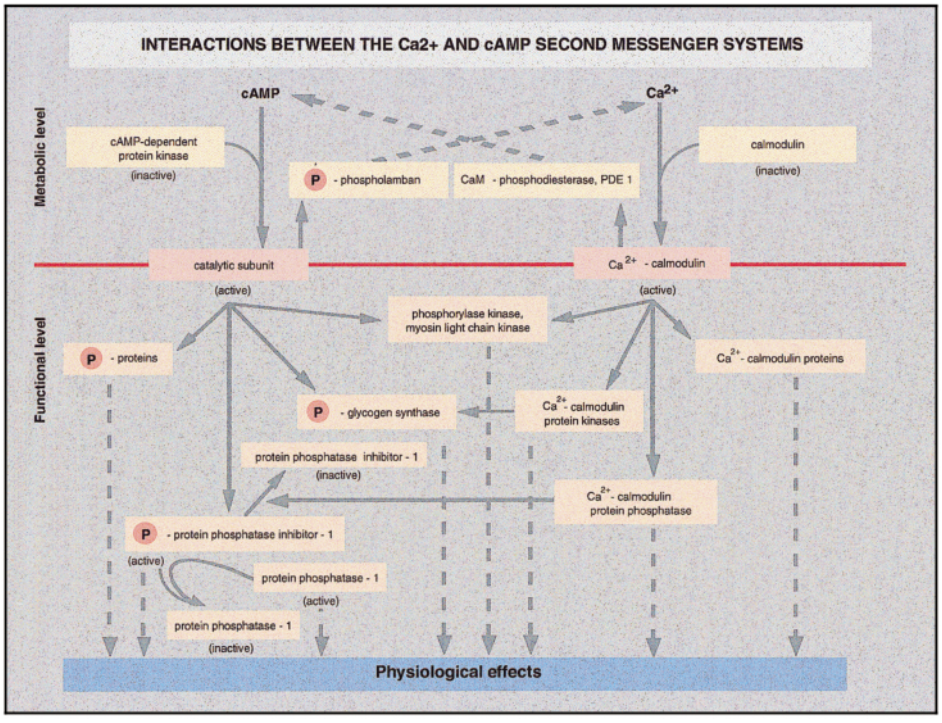

Figure 5. Schematic interactions between the cAMP and Ca^{2+} second messenger systems. The second messenger molecules cAMP and Ca^{2+} interact in several important fashions to integrate a physiological response. Some of these interactions are involved in the regulation of PDE1.

Table 4. Characterization of regulation of bovine PDE1 isozymes by phosphorylation and dephosphorylation.

Regulatory factor/characteristic	Isoenzyme		
	brain PDE1A2	brain PDE1B1	heart PDE1A1
Protein kinase	cAMP-dependent protein kinase	CaM-dependent protein kinase II	cAMP-dependent protein kinase
Stoichiometry	1 mol phosphate/mol subunit	1 mol phosphate/mol subunit	1 mol phosphate/mol subunit
	Decreases CaM affinity,	Decreases CaM affinity	Decreases CaM affinity
Effect on PDE	increases Ka of Ca^{2+} activation	increases Ka of Ca^{2+} activation	increases Ka of Ca^{2+} activation
Effectors	Ca^{2+}/CaM inhibits phosphorylation by binding to PDE1A2	Ca^{2+}/CaM does not inhibit phosphorylation	Ca^{2+}/CaM inhibits phosphorylation by binding to PDE1A1
Protein phosphatase	CaM-dependent phosphatase (calcineurin)	CaM-dependent phosphatase (calcineurin)	CaM-dependent phosphatase (calcineurin)

For details see [28, 59, 61].

Regulation of PDE1B1 by Ca^{2+} and CaM can occur by at least three mechanisms: (i) the PDE1B1 isozyme depends on Ca^{2+} and CaM for full activity, (ii) it requires higher concentrations of Ca^{2+} for activation upon phosphorylation by Ca^{2+} and CaM-dependent protein kinase(s) and (iii) the phosphorylation of PDE1B1 is reversed by the CaM-dependent protein phosphatase. Therefore, these three distinct CaM-dependent reactions can regulate PDE1B1 (table 4) and can produce opposite effects on PDE1B1. Based on our studies [61, 64], a working hypothesis has been proposed to indicate how these regulatory actions, separated temporally, could bring about meaningful interactions between the Ca^{2+} and cAMP signal systems during cell activation (fig. 6B). It is postulated that adenylate cyclase and the CaM-dependent protein kinase(s) can be turned on at lower concentration of Ca^{2+} during cell activation, whereas the activation of the PDE1B1 and CaM-dependent protein phosphatase require higher concentration of Ca^{2+}. The first two reactions, which act in concert to increase cAMP concentration, predominate at the early stage of surge in cytosolic Ca^{2+}, whereas the other two reactions will reduce cAMP concentrations at the higher concentra-

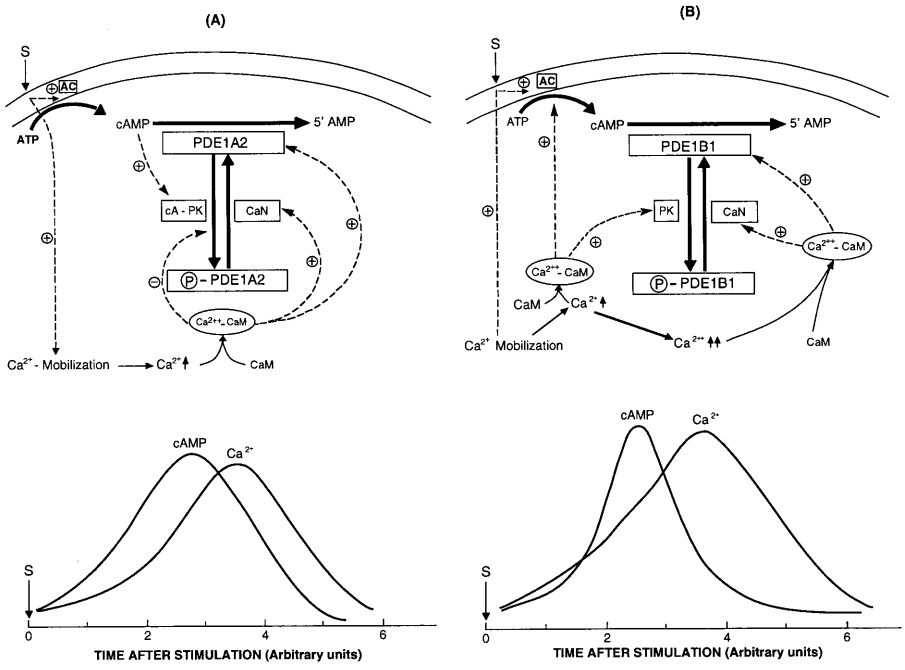

Figure 6. Hypotheses of the temporally separated regulation of PDE1A2 (*A*) and PDE1B1 (*B*) by Ca^{2+} and cAMP. Symbols: AC, adenylate cyclase; CaN, CaM-dependent protein phosphatase; cA-PK, cAMP-dependent protein kinase; PK, CaM-dependent protein kinase; P-phosphorylated; ⊕, activation; Θ, inhibition. Upper diagrams, organization of regulatory reactions; lower diagrams, simulated Ca^{2+} and cAMP fluxes; S, stimulus.

tions of the Ca^{2+} flux. For this proposed hypothesis, it is necessary that adenylate cyclase and the CaM-dependent protein kinase(s) be activated by lower concentrations of Ca^{2+} than the CaM-dependent protein phosphatase and PDE1B1 (fig. 6B). It has been reported that brain adenylate cyclase is indeed activated by much lower concentration of Ca^{2+} than the PDE1 [66, 67].

To test the validity of this working hypothesis depicted in figure 6B, we have purified and characterized CaM-dependent protein kinase from bovine brain. Bovine brain contains two CaM-dependent protein kinases which were separated on a Sephacryl S-300 column [63]. The high molecular weight, 500-kDa protein kinase has been purified close to homogeneity [63]. On the basis of its molecular mass, subunit size and protein substrate specificity, the purified bovine brain CaM-dependent protein kinase is considered to belong to the CaM-dependent protein kinase II family [63]. The phosphorylation of PDE1B1 by the CaM-dependent protein kinase II is dependent on the presence of Ca^{2+} and CaM (fig. 7A), and after phosphorylation a further increase in Ca^{2+} concentrations is required for enzyme activation (fig. 7B).

Earlier we postulated (fig. 6B) that the CaM-dependent protein kinase is activated by CaM at much lower concentrations of Ca^{2+} than the CaM-dependent protein phosphatase and PDE1B1 [61, 64]. However, this suggestion was not supported when the dose-dependent activation of PDE1B1 by Ca^{2+} was compared with the purified CaM-dependent protein kinase II at identical concentrations of CaM (fig. 8A vs. B). The results suggest that CaM-dependent protein kinase II and the PDE1B1 have similar Ca^{2+} concentration dependence at identical concentrations of CaM. However, the observation that the CaM-dependent protein kinase II becomes Ca^{2+}-independent and fully activated upon autophosphorylation suggests an alternative mechanism. The CaM-dependent protein kinase may use the autophosphorylation reaction to override its requirements for higher concentrations of Ca^{2+}.

Significance of autophosphorylation of calmodulin-dependent protein kinase II in the regulation of PDE1B1

Like other members of the CaM-dependent protein kinase family [68–73], this bovine brain CaM-dependent protein kinase II undergoes autophosphorylation rapidly [63, 74]. Some of the key features of the autophosphorylation reaction of CaM-dependent protein kinase II are presented in figure 9. The autophosphorylation reaction is completely dependent on Ca^{2+} and CaM. The reaction is rapid and completed with a maximal incorporation of about 1.0 mol phosphate per mol of subunit of CaM-dependent protein kinase II. Addition of excess EGTA after 1 min of autophosphorylation reaction had no effect on the autophosphorylation reaction when only 0.3 mol of phosphate per mol of subunit of CaM-dependent protein kinase II was observed (fig. 9A). These results indicated that the CaM-dependent protein kinase II rapidly converts to a totally Ca^{2+}-independent form by autophosphorylation. The PDE1B1 was phosphorylated by the autophosphorylated CaM-dependent protein kinase II, irrespective of the presence of Ca^{2+} or EGTA (fig. 9B). The slight

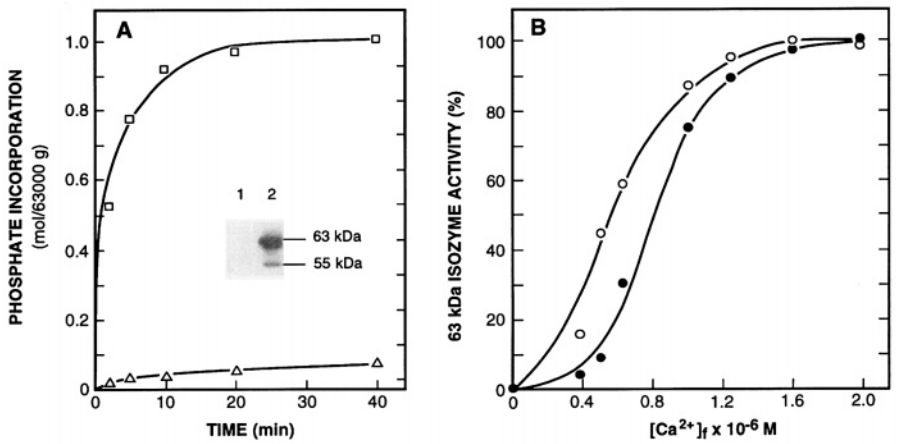

Figure 7. Phosphorylation of PDE1B1 by CaM-dependent protein kinase II. (*A*) Phosphorylation was carried out in the presence of Ca^{2+} (□—□) or EGTA (△—△). Inset autoradiograph: Lanes 1 and 2, PDE1B1 phosphorylated in the presence of CaM with 0.1 mM EGTA and 0.1 mM Ca^{2+}, respectively. (*B*) Effect of phosphorylation of the Ca^{2+} concentration dependence of PDE1B1 activation. Both nonphosphorylated (○—○) and phosphorylated PDE1B1 (●—●) were assayed for PDE1 activity. For experimental conditions, see Zhang et al. [63].

decrease in autophosphorylated CaM-dependent protein kinase II activity shown in figure 9B appears to be due to thermal inactivation of the enzyme. To test this possibility, the time course of autophosphorylation of the purified CaM-dependent protein kinase II at several Ca^{2+} concentrations was carried out (fig. 10). Maximal phosphorylation at suboptimal Ca^{2+} concentrations was much lower than 1.0 mol of phosphate per mol protein kinase subunit. A further addition of Ca^{2+} to a final concentration of 100 μM at 10 min failed to increase the level of phosphorylation (fig. 10). A possible explanation for the substoichometric phosphorylation is that the enzyme was labile under the incubation conditions. Furthermore, an accurate determination of the Ca^{2+} dose dependence of the autophosphorylation reaction is not possible, since the enzyme is apparently unstable at suboptimal concentrations of Ca^{2+}. However, both the rate and extent of phosphorylation of PDE1B1 by the autophosphorylated CaM-dependent protein kinase II are independent of Ca^{2+}, suggesting that binding of CaM does not change the substrate activity of PDE1B1.

The autophosphorylation of CaM-dependent protein kinase II requires Ca^{2+} and CaM to initiate the reaction to rapidly convert to a Ca^{2+}-independent form (fig. 9A), whereas the activation of PDE1B1 requires the continued presence of high concentrations of Ca^{2+} [22]. Therefore, the autophosphorylation of CaM-dependent protein kinase II, PDE1B1 phosphorylation and activity of PDE1B1 reactions were investigated in a single experiment, and the effect of EGTA on all three reactions was examined. Both autophosphorylation and phosphorylation of PDE1B1 became Ca^{2+}-independent after 15 s (fig. 11B,C). However, PDE1B1 activity was completely inhibited by the addition of EGTA (fig. 11A). These results suggest that the CaM-dependent protein kinase II is activated by a brief exposure to high concentrations of Ca^{2+}, whereas PDE1B1 requires the continued presence of high Ca^{2+} concentration for CaM activation.

There are a number of possible ways by which such a brief exposure of CaM-dependent protein kinase II to high concentrations of Ca^{2+} can occur at the onset of Ca^{2+} flux. Studies of Ca^{2+} transients in single cells have suggested that a Ca^{2+} surge may actually be composed of a series of rapid Ca^{2+} transients [75–77]. As a result, the CaM-dependent protein kinase II could be autophosphorylated and converted to a Ca^{2+}-independent form at the initial Ca^{2+} transient. Alternatively, it is possible that CaM-dependent protein kinase II may be localized proximal to Ca^{2+} entry sites and be briefly exposed to a high concentration of Ca^{2+} at the onset of a cell Ca^{2+} surge, resulting in autophosphorylation. Immunocytochemical studies have shown that CaM-dependent protein kinase II is localized at the inner surface of plasma membranes, as well as at the outer surface of mitochondria and at synaptic vesicles and microtubules [78]. Therefore, autophosphorylation of CaM-dependent protein kinase II may provide an additional mechanism that can be incorporated into a revised hypothesis for the regulation of PDE1B1 which is presented schematically in figure 12. For example, autophosphorylation of CaM-dependent protein kinase II can be reversed by protein phosphatase-1, which is regulated by protein phosphatase inhibitor-1 [79–82].

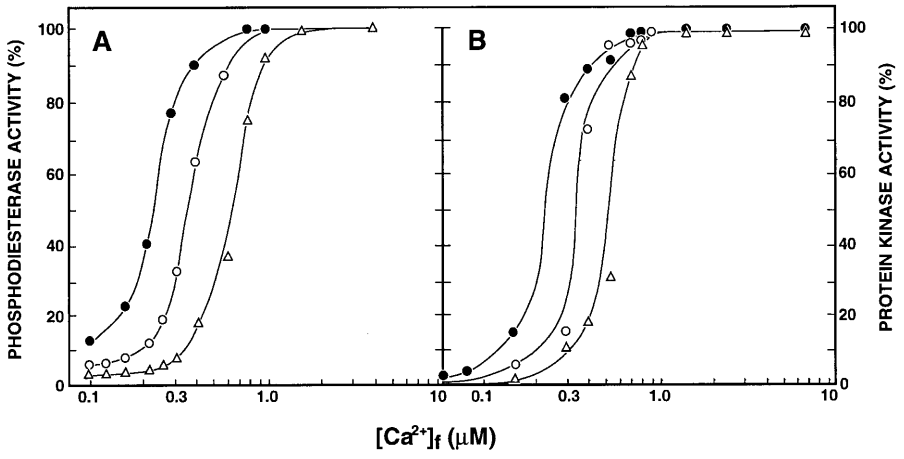

Figure 8. Comparison of activation of PDE1B1 with CaM-dependent protein kinase II at various concentrations of CaM. CaM-dependent protein kinase II and PDE1B1 assay were carried out by the procedure of Zhang et al. [63] and Sharma [22], respectively. CaM concentrations were 1 μM (△—△), 5 μM (○—○) and 20 μM (●—●). (*A*) PDE1B1 and (*B*) CaM-dependent protein kinase II.

When cAMP levels rise in the cell, cAMP-dependent protein kinase phosphorylates protein phosphatase inhibitor-1. Phosphorylated protein phosphatase inhibitor-1 is active and can inhibit the activity of protein phosphatase-1 [82, 83]. When protein phosphatase inhibitor-1 is dephosphorylated and thus inactivated by CaM-dependent protein phosphatase (calcineurin), protein phosphatase-1 is reactivated. As a result, cAMP may exert an inhibitory effect on PDE1B1 isozyme through a regulatory cascade involving protein phosphatase inhibitor-1, protein phosphatase-1 and CaM-dependent protein kinase II. Furthermore, the cAMP effect can be reversed by CaM-dependent protein phosphatase through dephosphorylation of phosphorylated protein phosphatase-1. This complex regulatory interaction (fig. 12) is in agreement with the previously suggested role for PDE1B1 isozyme in the dynamic coupling of cAMP and Ca^{2+} fluxes in the cell [4, 5, 64]. In summary, during the early stage of cell activation, the initial increase in cAMP and Ca^{2+} cause a temporary suppression of PDE1B1 activity to maintain the rise in cAMP concentration. As the Ca^{2+} concentration in the cell is subsequently elevated, the CaM-dependent protein phosphatase is activated to reverse phosphorylation of the PDE1B1 and reactivates the PDE1B1. Since CaM-dependent protein phosphatase also dephosphorylates protein phosphatase inhibitor-1 to cause the reactivation of protein phosphatase-1, autophosphorylation of CaM-dependent protein kinase II is also reversed; therefore, rephosphorylation of PDE1B1 will no longer occur as the Ca^{2+} concentration subsides in the cell.

Significance of the proteolysis of PDE1 in the regulation of cAMP concentration PEST motif in brain PDE1A2 sequence

It has been proposed that polypeptide sequences enriched in proline (P), glutamate (E), serine (S) and threonine (T), known as PEST motifs, serve as putative intramolecular signals for rapid proteolytic degradation by specific proteases [84]. Computer analysis has shown that PEST motifs are found in most proteins known to be metabolically unstable or expected to be so, but rarely in stable proteins [84, 85]. PEST sequences vary in length from 12 to 100 amino acids and can reside anywhere along the molecule. It has been proposed that proteins which contain PEST sequences may increase the local calcium concentration, and serve as a recognition signal for Ca^{2+}-dependent cysteine proteases (calpains) [84, 86]. The strength of a PEST sequence is determined by the PEST score, which range from -45 to $+50$. A PEST score of <0 but >-5 represents a weak PEST region, and a PEST score of >0 denotes a possible PEST region, but a value $>+5$ indicates a very strong PEST motif [87]. The amino acid sequence of bovine brain PDE1A2 derived from cDNA sequence (GenBank accession number M90358) [88] was used to identify PEST motifs by the PEST-FIND program [84] using the website www.icnet.uk/cgi-bin/runpest.pl. The sequence analysis revealed that there is only one PEST motif from N-terminal residue 73–94 (RLLDTDDELSIQSDDSVPSEVR) with a PEST score of 7.36. Numerous proteins with PEST motifs are susceptible to proteolysis by calpain in vitro [87]. However, other reports have not supported any causal relationship be-

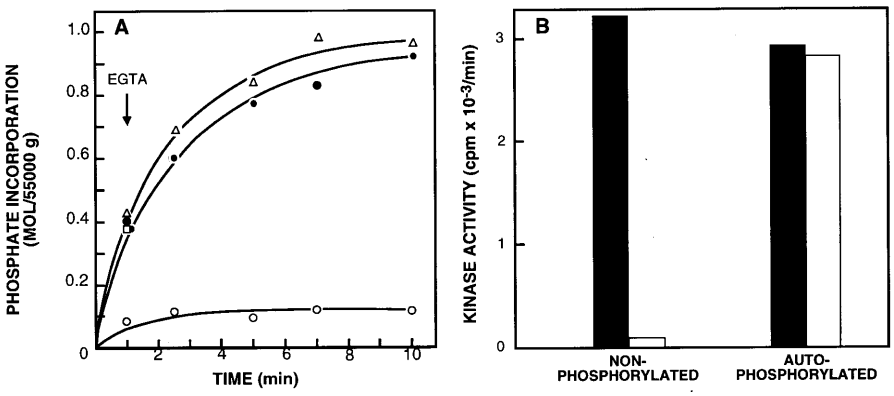

Figure 9. Characterization of autophosphorylation of CaM-dependent protein kinase II. (*A*) The autophosphorylation reaction of CaM-dependent protein kinase was carried out in the presence of Ca^{2+} and CaM (△—△, ●—●). After 1 min (●—●) excess EGTA was added as indicated by arrow. Autophosphorylation reaction was also carried out in the presence of EGTA and CaM (○—○). (*B*) Conversion of CaM-dependent protein kinase II to a Ca^{2+}/CaM-independent form by autophosphorylation. The autophosphorylation of CaM-dependent protein kinase II was carried out for 5 min in the presence of unlabelled ATP, CaM and Ca^{2+}. Control CaM-dependent protein kinase II (nonphosphorylated) was also incubated without unlabelled ATP. After autophosphorylation, the activities of nonphosphorylated and autophosphorylated CaM-dependent protein kinase were determined by using PDE1B1 in the presence of CaM either Ca^{2+} (■) or EGTA (□). For experimental conditions, see Zhang et al. [63, 74].

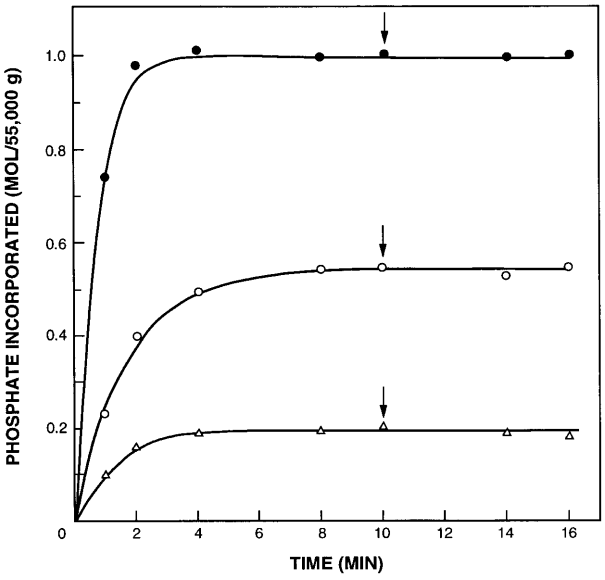

Figure 10. Effect of Ca^{2+} on the autophosphorylation of CaM-dependent protein kinase II. Autophosphorylation reaction of CaM-dependent protein kinase II was carried out at various concentrations of free Ca^{2+}, 0.08 μM (△—△), 0.4 μM (○—○) and 100 μM (●—●) in the presence of CaM. After 10 min, Ca^{2+} was added to a final concentration of 100 μM as indicated by the arrow. For details, see Zhang et al. [63].

tween PEST motifs and calpain recognition. In the cases of c-Fos protein and the plasma membrane Ca^{2+}-ATPase, mutations lowering the PEST score do not influence the susceptibility of the enzyme to calpains [85, 89]. Recently, we identified that heart PDE1A1 (R. Kakkar and R. K. Sharma, unpublished results) and brain PDE1A2 have PEST motifs and act as substrates for m-calpain. However, the cleavage site is far from the PEST motif [90]. In this review we describe our studies on PDE1A2.

Activation of PDE1A2 by m-calpain

Calpains function as one of the major mediators for calcium signals in many biological systems [91–93]. Two forms of calpain, which differ in their Ca^{2+} requirements, have been identified: those requiring micromolar concentration are known as μ-calpains, and those requiring millimolar concentration are known as m-calpains [91]. However, both enzymes undergo Ca^{2+}-dependent autolysis, which greatly increases their sensitivity to Ca^{2+}. The Ca^{2+} requirement of m-calpain is reduced to the micromolar range in the presence of phospholipids, and it has been reported that μ-calpain activates m-calpain [94]. The reduction in Ca^{2+} sensitivity of m-calpain has potential physiological significance for its regulation. Both μ- and m-calpain and their endogenous inhibitor, calpastatin, are ubiquitously expressed in different tissues and play an important role in various physiological and pathological processes [91–100]. In brain, calpains are implicated in synaptic modification, neurite pruning, receptor characteristics, neurofilament turnover and neural differentiation [92, 99, 100]. Several reports indicate that calpains are involved in axonal neurofilament degradation, motor-neuronal degradation, neuronal ischemia and other neurodegenerative diseases, including Alzheimer's and epilepsy [91, 92, 99–101]. This has been attributed generally to the irreversible proteolysis of a wide range of substrates, in particular cytoskeletal and membrane

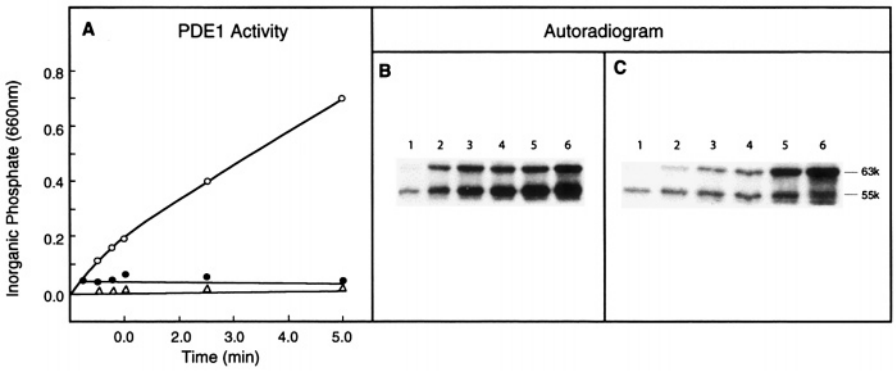

Figure 11. Effect of EGTA on the autophosphorylation of CaM-dependent protein kinase II, phosphorylation of PDE1B1 and PDE1B1 activity. The reaction mixture contained PDE1B1, CaM-dependent protein kinase II, CaM, 5′-nucleotidase and cAMP. The reaction was carried out in the presence of either 0.1 mM Ca^{2+} (○—○, ●—●) or 0.1 mM EGTA (△—△) and [γ-^{32}P] ATP. After 15 s, EGTA was added to a final concentration of 1 mM to one of the reactions containing 0.1 mM Ca^{2+} (●—●). At various time intervals indicated, aliquots were removed for the PDE1B1 activity and for the SDS-PAGE and autoradiography. (*A*) PDE1B1 activity; (*B*) autoradiograph of the Ca^{2+}-containing reaction (○—○); and (*C*) autoradiograph for the fraction with EGTA added after 15 s (●—●). For lanes 1–6, aliquots were taken at 0.25, 0.5, 0.75, 1.0, 2.5 and 5.0 min for SDS-PAGE and autoradiographic analysis. For details, see Zhang et al. [63].

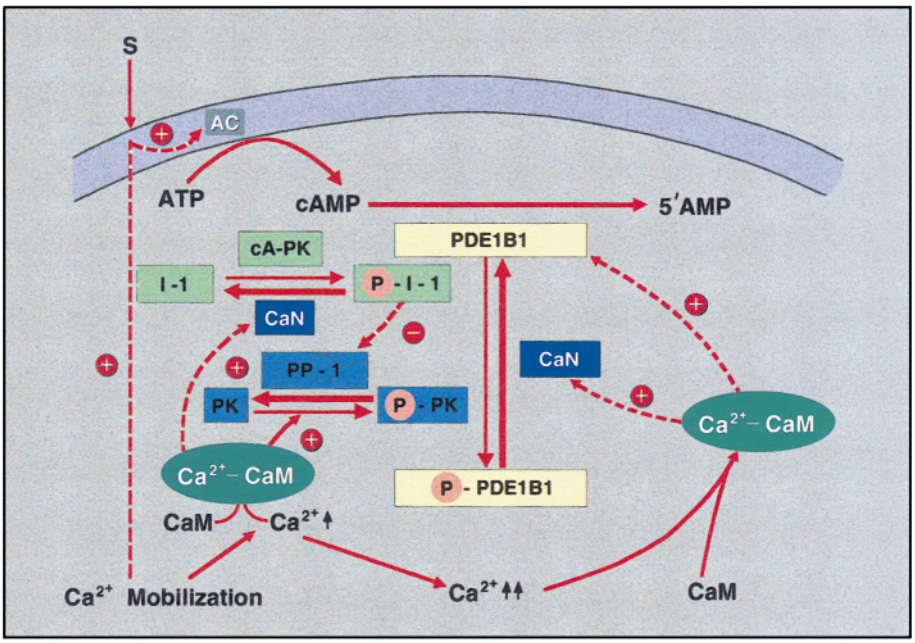

Figure 12. Schematic diagram of the regulation of PDE1B1 by Ca^{2+} and cAMP as mediated by the autophosphorylation mechanism of CaM-dependent protein kinase II. The scheme depicts the complex interactions among: cA-PK, cAMP-dependent protein kinase; PP-1, protein phosphatase-1; I-1, protein inhibitor-1; P-phosphorylated; CaN, CaM-dependent protein phosphatase; ⊕, activation; Θ, inhibition; light arrow, early events, and dark arrow, late events.

proteins, various enzymes, cytokines and transcriptional factors (Myc, c-Fos, c-Jun, p53 and NF-kB) [90, 91, 98–103].

An increase in the intracellular concentration of Ca^{2+} can result in the activation of CaM-dependent enzymes, including Ca^{2+}-dependent proteases. PDE1 serves as an important component of the calcium-dependent signal transduction pathway that modulates physiological responses mediated by cyclic nucleotides [2, 90, 104–107]. Proteolytic cleavage of PDE1A2 by m-calpain generated a 45-kDa immunoreactive fragment which is an active, CaM-independent form and a 15-kDa fragment (not shown) which did not show any immunoreactivity with PDE1A2 antibody, suggesting that it lacks the antigenic epitope (fig. 13A) [90]. Addition of more m-calpain to the proteolytic reaction resulted in an insignificant loss of the 45-kDa fragment, suggesting that it is resistant to further proteolysis. The time course of conversion of PDE1A2 into a CaM-independent form is shown in figure 13B. The presence of CaM in the proteolytic reaction did not have any effect on PDE1A2 activity, suggesting that the interaction between CaM and PDE1A2 does not alter substrate recognition by calpain [90]. It has been reported that some CaM-binding domains, such as Ca^{2+}-adenosine triphosphate (ATPase), may be a preferred substrate for calpain [108]. Hydrophobic interactions are critical for calpain substrate interaction, and this is supported by the interaction between calpains and their inhibitor calpastatin [97, 98]. Calpastatin did not alter PDE1A2 activity, but it did inhibit calpain-mediated proteolysis [90]. A CaM overlay using biotinylated CaM revealed that the 45-kDa fragment does not contain the CaM binding site and suggested that the cleavage site is not in the CaM binding domain [90]. Furthermore, the phosphorylation of PDE1A2 by cAMP-dependent protein kinase and treatment with m-calpain resulted in generation of the 45-kDa fragment, suggesting that phosphorylation does not protect PDE1A2 from calpain action. A previous study on connexin-32 indicated that its phosphorylation by protein kinase C prevents proteolysis by calpains [109]. It has been shown that two serine residues of PDE1A2 are phosphorylated by cAMP-dependent protein kinase, at serine 112 and serine 120 [60]. Our results suggested that the calpain cleavage site could be distant from the phosphorylation site. The cleavage site for calpains rarely resides in the PEST motif [85], although in some calpain substrates PEST sequences are located near the cleavage site. Brain PDE1A2 has a single PEST motif (residues 73–94), and calpain cleaves PDE1A2 after residue 126, which is 32 residues away from the PEST motif. For various CaM-binding proteins, there is a high degree of correlation between the location of the CaM binding site and PEST motifs [86]. Wang et al. [108] suggested that CaM-like calcium

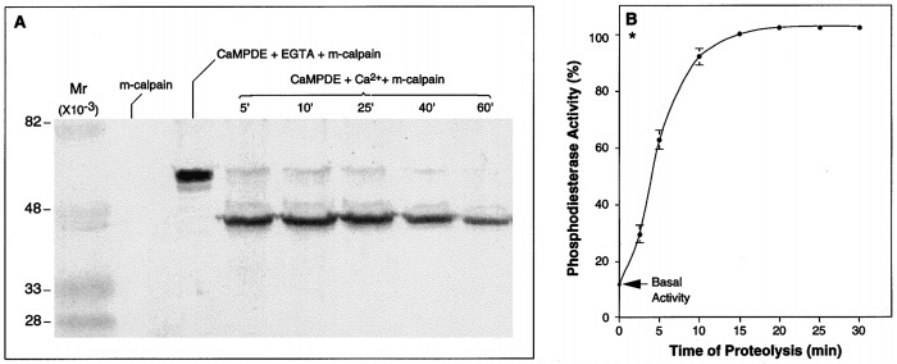

Figure 13. (*A*) Time course of brain PDE1A2 proteolysis by m-calpain. SDS-PAGE was carried out, and proteins were transferred onto nitrocellulose membrane and probed with brain PDE1A2 antibody. (*B*) Effect of proteolysis on PDE1A2 activity. PDE1A2 was incubated with m-calpain in the presence of Ca^{2+}, and at the times indicated proteolytic reaction was stopped with leupeptin and aliquots were assayed for activity in the presence of 5 mM EGTA. In the figure, the arrow indicates the basal enzyme activity, in the presence of EGTA and absence of m-calpain; * indicates the activity of fully activated PDE1A2 in the presence of Ca^{2+}/CaM and absence of m-calpain after 30 min; the activity was taken as 100%. For details see Kakkar et al. [90].

binding domains of calpain participate in substrate recognition by association with the CaM binding domains of CaM-regulated proteins.

The domain organization of the PDE1A2 revealed that the native protein is a 530-residue polypeptide, a homodimer of N^{α}-acetylated, and is composed of separate catalytic and regulatory domains [88, 110]. From the N-terminus, the CaM binding site is located from residue 23–41, the PEST motif from 73–94 and the phosphorylation site from 110–120, respectively. The catalytic domain is comprised of residues 139–446. It encompasses an approximately 250-residue sequence (193–446) which is conserved among PDE isozymes of diverse size, phylogeny and function [2]. Our analysis suggested that the molecular weight of this domain was 45 kDa, suggesting that the fragment is comprised of residues 139–530, when the enzyme is converted into a CaM-independent form. In view of the other domains, the most probable site for calpain cleavage could be between residues 120 and 138. This was confirmed by the N-terminal sequence analysis of the 45-kDa fragment, which indicated that the calpain cleavage occurs between residues ^{126}Glutamine and ^{127}Alanine [90] (fig. 14).

Calpains cause limited proteolysis of substrates, resulting in modification of activity rather than inactivation or complete degradation. For example, protein kinase C, after cleavage by calpain, becomes fully active but no longer requires Ca^{2+} and phospholipid for activation [111]. Several structural proteins such as neurofilament, spectrin, microtubule-associated protein, *Tau* and calcium channels act as substrates of calpains, which may be important for modification of neuronal morphology [92, 99, 100, 112]. Alterations in intracellular Ca^{2+} homeostasis and activation of calpains have been implicated in various neurodegenerative disorders [92, 100, 112, 113]. Recently, Jordan et al. [114] showed that calpains play a key role in β-amyloid-induced apoptosis of hippocampal neurons, which is blocked by overexpression of the Ca^{2+}-binding protein calbindin D28K [115]. α-Spectrin is cleaved by both calpains and ICE-like proteases into distinct fragments during apoptosis in neuronal cells, and inhibition of these proteases prevents neuronal apoptosis [116, 117]. These findings suggest that agents that act as calpain inhibitors may be effective in a variety of neurological disorders.

Our findings suggest that the proteolysis of PDE1A2 by m-calpain results in a CaM-independent form which in turn could decrease the intracellular levels of cAMP [90]. The potential role of PDE1 isozymes in the pathogenesis of certain neurological conditions remains an important area of investigation. However, there is considerable evidence that cAMP is involved in various neuronal functions, including synaptic transmission and neuron survival [104–107]. It has been reported that in patients having Parkinson's disease with dementia, there is a significant decrease in cAMP [118]. We have demonstrated that PDE1A2 is inhibited by antiparkinsonian agents, suggesting a potential role of PDE1 in Parkinson's disease [119, 120]. Since PDE1A2 is predominantly expressed in brain [2, 106], its colocalization with calpains in the same regions which contain D_1 and D_2 receptors can affect the cAMP signal. In certain pathophysiological conditions, an increase in Ca^{2+} influx as a result of inositol trisphosphate generation or via stimulation of glutamate receptors would attenuate the duration and magnitude of the cAMP signal. This could occur either by activating PDE1 and/or by activating calpain, which in turn can cleave PDE1, making

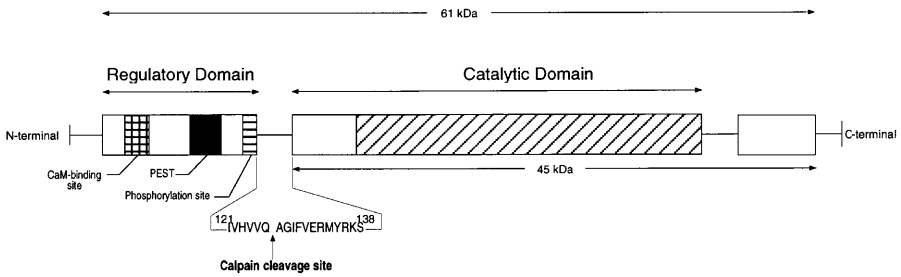

Figure 14. Cleavage site for m-calpain-mediated truncation of PDE1A2. The primary sequence of PDE1A2 was adapted from Sonnenburg et al. [88]. The figure indicates regulatory and catalytic domains. The square, solid black, horizontally hatched and diagonally hatched boxes represent the CaM binding domain, PEST motif, phosphorylation site and conserved catalytic domain, respectively. The arrow denotes the m-calpain cleavage site between residues ^{126}Gln and ^{127}Ala. For details see Kakkar et al. [90].

it independent of CaM. Since PDE1A2 also has an affinity for cGMP [40], calpains can also affect the cGMP signal. The activation of PDE1 by calpains could be an alternative physiological mechanism to regulate cAMP concentration in the cells by activating enzymes which are otherwise regulated reversibly by Ca^{2+}/CaM. It may be particularly important in neurons, which undergo cyclical changes in intracellular Ca^{2+} during neurotransmission as well as for neuronal gene expression. The regulation of PDE1A2 by m-calpain may provide an important route for controlling intracellular levels of cAMP and the physiological processes regulated by these messenger molecules.

PDE1 in tumor tissue

PDEs have been studied in a variety of tumors [121–126], and Sharma and Hickie have summarized these results previously [1]. The results from these studies in non-central-nervous system tumors suggest that the levels of PDE activity are elevated and thereby affect the ratio of cGMP to cAMP. In addition, PDEs can also be considered as potential targets for cell growth inhibition [1, 122, 124]. However, very little is known about the involvement of individual PDE isozymes in the various tumor tissues. Various reports have indicated that levels of PDE1 are elevated in tumors [122–126]. However, recently Lal et al. [127] reported that catalytic activity of PDE1 in glioblastoma multiforme was significantly reduced compared with normal cerebral cortex. In addition, it was also observed that the more malignant the tumor, the more significantly reduced the catalytic activity of PDE1 (S. Lal and R. K. Sharma, unpublished results). Furthermore, normal human temporal cortical neurons and dendrite/axonal endings demonstrate strong cytoplasmic labelling using A6 monoclonal antibody, whereas glioblastoma cells failed to react effectively with the antibody (fig. 15A vs. B) [127]. However, entrapped neurons in this sample showed some immunoreactivity. Therefore, this study suggested that the proliferation of cerebral tumors may be different than that of other types of tumors. In addition, a heat-labile inhibitor protein for PDE1 was also discovered from patients with glioblastoma multiforme and glioblastoma

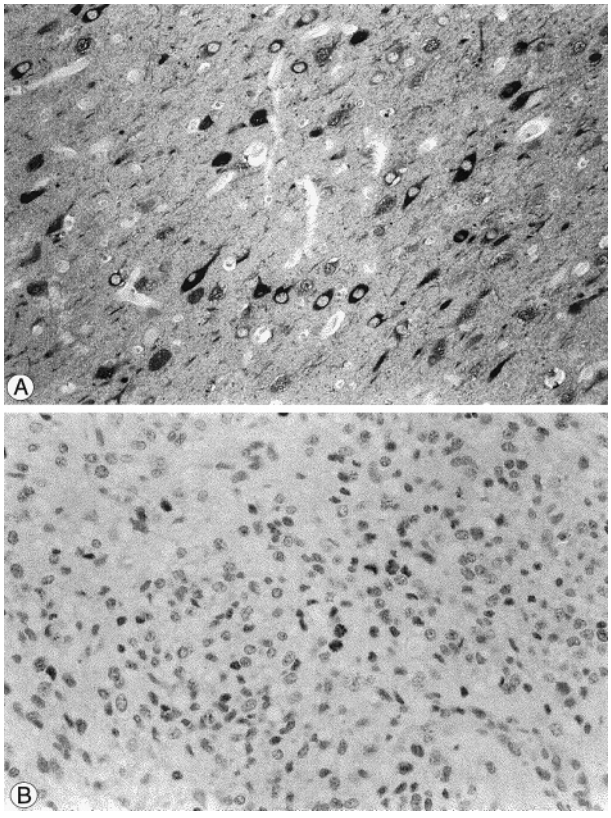

Figure 15. (*A*) Temporal cortical tissues demonstrating positive staining for the PDE1A2 in the cytoplasm and in the axonal process of many neurons (magnification 400 ×). (*B*) Glioblastoma multiforme section demonstrating absence of staining with the antibody for PDE1A2. No tissue necrosis was present in this region of the tumor (magnification 250 ×).

[128]. Sephacryl S-200 gel filtration column chromatography indicated that the inhibitor had an apparent molecular weight of 22 kDa [128]. Further work is required to help in better understanding of the function of PDE1 in tumors of the central nervous system.

Inhibitors of PDE1 isozymes

The importance of cyclic nucleotides as second messengers is well established in various physiological processes [104–107, 129, 130]. More than 30 PDEs representing seven or more families exist in humans. Because PDEs exist in multiple forms in cells and tissues, with each form acting in a complex manner in various responses, selective PDE inhibitors are very useful tools in investigating the roles of PDEs in those responses. The clinical trials for several selective PDE inhibitors of PDE1, PDE3, PDE4 and PDE5 families have been carried out or are in progress [2, 131]. A number of drugs are in clinical trials for asthma, and Viagra has become the first selective PDE inhibitor to be approved by the U.S. Food and Drug Administration [131]. Agents like theophylline are still used in the treatment of asthma and are also used as an adjunct in several other disorders [132]. Pentoxifylline is a nonselective PDE inhibitor which has proved effective for a variety of disease states [133]. Cilostazol is one of such drugs able to be used clinically as a novel antithrombotic drug with a potential to inhibit platelet aggregation [129]. The in vivo efficacy of most of the PDE inhibitors may not necessarily match their in vitro potency due to issues of permeability, distribution and metabolism.

The purpose of this section is to summarize briefly the recent developments by which the activity of individual PDE1 isozymes is inhibited by various pharmacological agents. Several aspects of PDE1 inhibitors have been reviewed previously [1, 2, 4]. Early studies on inhibitors were carried out by using purified or partially purified PDE1. In addition, it was not clear from previous studies which of the purified or partially purified PDE1 isozymes were used. Our laboratory has demonstrated that PDE1 isozymes may be distinguished by several pharmacological agents. The IC_{50} (half maximal inhibition) values for the inhibition of individual isozymes by various pharmacological agents are summarized in table 5. Ginsenosides, used for the treatment of heart failure and which also have antipsychotic, anticonvulsant and antifatigue actions [134, 135] were found to be potent inhibitors of heart PDE1A1 and brain PDE1A2 but not of brain PDE1B1 [136]. However, deprenyl (selegeline hydrochloride), an antiparkinsonian agent which is an inhibitor of monoamine oxidase-B, also inhibits brain PDE1A2 but is a poor inhibitor of brain PDE1B1 [119]. In addition, we also studied amantadine, which is used for the treatment of Parkinson's disease and for other disorders including stroke, epilepsy and neuroleptic malignant syndrome [137]. It is interesting to note that amantadine only inhibits brain PDE1A2 isozyme but not brain PDE1B1, heart PDE1A1 and lung PDE1 isozymes [120]. Since the inhibition of these isozymes is overcome by increasing the concentration of CaM, this suggests that these compounds act specifically and reversibly against the action of CaM. Therefore, these compounds may be valuable tools to investigate the diverse physiological roles of distinct PDE1 isozymes.

Unlike other CaM-dependent enzymes, PDE1 has been suggested to be inhibited by dihydropyridine calcium antagonists, which act as direct vasodilator drugs [138–141] rather than indirectly through their actions on CaM [142–146]. In previous studies, it was not clear which of the specific PDE1 isozymes were used [147–150]. We have examined the effect of dihydropyridine Ca^{2+} channel blockers felodipine and nicardipine on purified brain PDE1 isozymes [151]. The results indicated that both brain isozymes are inhibited by felodipine and nicardipine by partial competitive inhibition, and these two Ca^{2+} antagonists appear to counteract each other [151]. The K_i values for felodipine (1.8 and 2.8) and nicarpidine (2.3 and 5.8) for PDE1A2 and PDE1B1, respectively, suggest that the two brain PDE1 isozymes have similar affinities for the Ca^{2+} antagonists, and both the isozymes bind felodipine slightly tighter than they bind nicarpidine. This study further demonstrated the existence of a specific site, distinct from the active site on PDE1 isozyme, which exhibits high-affinity binding of these drugs. The pharmacological significance of these inhibitors requires further research.

Cyclic nucleotides and Parkinson's disease

Idiopathic Parkinson's disease is an irreversible neurodegenerative disorder affecting extrapyramidal function. The neurochemical pathology of Parkinson's disease is loss of neurons in the substantia nigra, leading to deficiency of dopamine in the striatum [152]. The clinical efficacy of dopamine replacement therapy depends on the preservation of postsynaptic dopamine receptors and their intracellular signalling machinery in the striatum [153, 154]. Besides Ca^{2+}, dopamine constitutes a major signalling pathway in striatal neurons. It activates adenylate cyclase and phospholipase C via a D_1 receptor and inhibits through D_2 receptors, thereby regulating the production of intracellular second messengers cAMP and 1,2-diacylglycerol coupled with the release of Ca^{2+} from intracellular stores [155–157].

Table 5. Half maximal inhibition (IC_{50}) of various pharmacological agents on the activity of PDE1 isozymes.

Isozyme	Pharmacological agents				
	Ginsenoides (μM)			Amantadine (μM)	Deprenyl (mM)
	Rc	Rb	Re		
Heart PDE1A1	3.7	6.8	14.8	nd	nc
Brain PDE1A2	3.7	6.3	12.7	4.8	1.0
Brain PDE1B1	nd	nd	nd	nd	2.0
PDE1C	nc	nc	nc	nd	nc

For details see [119, 120, 136]. nc, not carried out; nd, not detected.

Alteration in dopamine receptors has been studied extensively in postmortem brain tissues of Parkinson's disease patients, and data have been controversial [118, 152–154, 158]. Nishino et al. [118] have reported that cAMP levels are decreased in brains of demented Parkinson's disease patients. PDEs are essential regulators of cyclic nucleotides, and to our knowledge, there has been no documentation of changes in PDE levels in Parkinson's disease. The relevance of PDE isoforms in Parkinson's disease remains unknown.

It has been reported that in isolated brain slices and preganglionic nerve fibres, stimulation with dopamine produced a severalfold increase in cAMP levels in the cervical sympathetic ganglia which was associated with synaptic transmission [159, 160]. Nerve growth factor (NGF) is a polypeptide and important mediator for nervous system development. It has been reported that cAMP analogs 8-(4-chlorophenylthio)-cAMP and 8-bromo cAMP can replace NGF in survival and neurite outgrowth in cultures of rat neonatal sympathetic and sensory neurons [161, 162]. It has also been reported that neuronal death produced by electrical blockade and decreased cAMP levels with tetrodotoxin (TTX) was attenuated by the addition of 8-bromo-cAMP, whereas 8-bromo-cGMP under the same conditions had no protective effects [162]. Hartikka et al. [163] have demonstrated that increased intracellular levels of cAMP enhance the dopamine uptake per neuron and protect mesencephalic-dopaminergic neurons from neurotoxin MPP^+-induced degeneration.

Deprenyl ameliorates the symptoms of Parkinson's disease in humans as well as 1-methyl-4-phenyl-1,2,3,6-tetrahydroxy pyridine (MPTP)-induced animal models [164]. It has been proposed that it acts as an irreversible inhibitor of monoamine oxidase-B, which enhances dopaminergic transmission in brain, acts as a glutamate receptor antagonist, and potentiates the antiparkinsonian action of L-dopa [164–166]. It has also been reported that deprenyl reduces pheochromocytoma PC12 cell apoptosis and alters protein synthesis and gene expression [167]. On the other hand, amantadine ameliorates the symptoms of Parkinson's disease in humans, as well as in animal models [137, 168–170], and acts possibly by N-methyl-D-aspartate receptor antagonism [137, 169]. It acts presynaptically to enhance dopamine release or inhibit dopamine uptake [171]. We have demonstrated that deprenyl may be a more specific inhibitor of PDE1A2 than of PDE1B1; however, amantadine inhibits only PDE1A2 but not the other isozymes. The IC_{50} value of amantadine for PDE1A2 was 4.75 μM. This concentration is in agreement with the previously reported values for amantadine (5–30 μM), in which a neuroprotective effect has been shown in vivo [172, 173]. Weller et al. [173] described neurotoxic effects of amantadine at concentrations of 100–200 μM in cerebella, cortical and mesencephalic neurons.

PDE1A2 is predominantly expressed in brain [2, 106], and its inhibition by deprenyl and amantadine can lead to enhanced intracellular levels of cAMP. There is considerable evidence that cAMP is involved in the regulation of metabolism and function in nervous system and neuronal survival [159–163, 174]. Brenneman et al. [162] reported that neuron survival is possible only when cAMP levels are maintained within a critical range. Excessive cAMP, as with prolonged exposure to high doses of exogenous cAMP derivative, or limited cAMP, as with TTX treatment, results in neuronal death. The compounds which increase intracellular cAMP may protect dopaminergic neurons under stress. Our findings suggest that inhibition of PDE1 isozymes by these agents can lead to increased intracellular levels of cAMP, which may be one of the contributing factors for increased survival of neurons. However, the mechanism by which increased cAMP could lead to an increase in neuronal survival is unknown. The cAMP may produce long-lasting physiological effects on synaptic membranes through activation of cAMP-dependent protein kinase and consequent phosphorylation of proteins in synaptic membranes [161]. Lewis et al. [175] have reported that in rat PC12 cells cAMP increases tyrosine hydroxylase activity and messenger RNA

(mRNA) levels, which could be a putative mechanism for increased dopamine synthesis. The cAMP-dependent protein kinase regulates the survival and differentiation of dopaminergic substantia nigra neurons in vivo and in vitro, implicating a therapeutic potential for substances which regulate cAMP turnover in these neurons [163]. It has been shown that increasing intracellular cAMP with PDE type IV-specific inhibitors enhanced the survival of dopaminergic neurons and reduced the MPTP-induced dopamine depletion in the striatum of mice [176]. One of the endogenous substrates for cAMP-dependent protein kinase is DARPP-32 (dopamine and cAMP-regulated protein, 32 kDa), which is abundant in striatum. Its phosphorylation is linked to activation of dopamine receptors and regulation of dopaminergic transmission [177–179]. Various genes are regulated through a conserved cAMP response element (CRE). A nuclear CRE-binding protein, CREB, binds to the CRE and stimulates the transcription of cAMP-responsive genes which can alter neuronal function by altering gene expression [105, 177].
Further studies of region-specific alterations of second messenger systems in the brain of Parkinson's disease patients and comparison with experimentally induced parkinsonism should explain the role of second messenger molecules in Parkinson's disease. This may lead to a more effective strategy for the treatment of Parkinson's disease and other disorders such as schizophrenia and neuropsychiatric disorders involving abnormal dopaminergic functions. Intracellular signalling systems are complicated, but these multiple sites for pharmacological intervention provide a basis for their mechanism of action and development of new drugs.

Conclusions

PDE1 exists in different isoforms which exhibit distinct molecular and/or catalytic properties. The kinetic, activation and regulatory characteristics have revealed subtle differences between these isozymes. Although PDE1 isozymes are regulated by Ca^{2+} and CaM, their stimulation by these activators is differentially modulated by other posttranslational modifications. Recently, it has been reported that PDE1 isozymes have PEST motifs and act as substrates of calpains. This provides a new insight into the regulation of PDE1 independent of CaM. The alterations in intracellular Ca^{2+} concentration dictates the activity changes of various CaM-dependent enzymes, including PDE1, which consequently determines the change in the intracellular cyclic nucleotide level. Various studies indicate that the activity of PDE1 is precisely regulated by cross-talk between the Ca^{2+}- and cAMP-signalling pathways. This review describes the range of complex interactions between cAMP and other signalling systems and the likely pivotal role of distinct PDE1 isozymes in controlling cAMP levels. Furthermore, these studies suggest that PDE1 isozymes may be useful targets for therapeutic intervention with respect to disorders of the central nervous system and the cardiovascular system. However, there is a lack of apparent progress with respect to development of isozyme-specific PDE1 inhibitors, which means that the functional role of PDE1 isozymes remains speculative. The various splice variants of PDE1 pose challenges to future pharmacological studies and the development of specific inhibitors. As reviewed in this article, PDE1 isozymes are key components of signal transduction pathways. Despite substantial progress, we must acknowledge that our understanding of the function of PDE1 in disease states is far from complete. Indeed, in the future it will be important to identify adaptive responses where PDE isozyme activity and expression are changed, and the changes in levels of proteins which regulate and target PDE isoforms. This will provide a basis for the development of novel therapeutic agents that are specific for the individual isozymes.

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