Regulation of calmodulin-stimulated cyclic nucleotide phosphodiesterase (PDE1): Review

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Abstract. The response of living cells to change in cell environment depends on the action of second messenger molecules. The two second messenger molecules cAMP and Ca^{2+} regulate a large number of eukaryotic cellular events. Calmodulin-stimulated cyclic nucleotide phosphodiesterase (PDE1) is one of the key enzymes involved in the complex interaction between cAMP and Ca^{2+} second messenger systems. Some PDE1 isozymes have similar kinetic and immunological properties but are differentially regulated by Ca^{2+} and calmodulin. Accumulating evidence suggests that the activity of PDE1 is selectively regulated by cross-talk between Ca^{2+} and cAMP signalling pathways. These isozymes are also further distinguished by various pharmacological agents. We have demonstrated a potentially novel regulation of PDE1 by calpain. This study suggests that limited proteolysis by calpain could be an alternative mechanism for the activation of PDE1. We have also shown PDE1 activity, expression and effect of calpain in the rat model *in vitro* of cardiac ischemia-reperfusion.

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1. Introduction

A variety of cellular activities are regulated through mechanisms controlling the level of cyclic nucleotides. These mechanisms include synthesis, degradation, efflux and sequestration of cyclic adenosine 3':5'-monophosphate (cAMP) and cyclic guanosine 3':5'- monophosphate (cGMP) within the cell. Hydrolysis of the cyclic nucleotides by cyclic nucleotide phosphodiesterases (PDEs) is a unique mechanism for the degradation of cyclic nucleotides. The PDEs isolated from various tissues differ in their substrate specificities, in their substrate affinities and in their sensitivity to a variety of natural and artificial effectors. The variability has been observed even among the enzymes isolated from a single tissue. The presence of multiple forms of PDE within a single tissue or cell type has been a consistent finding.

Initially, the multiple forms of PDE were classified into three categories: cAMP PDE, cGMP PDE and PDEs based on their marked differences in relative affinities towards cyclic nucleotides (1,2). One of these forms of enzymes is activated by Ca^{2+}/calmodulin (CaM) and has a lower apparent K_m value for cGMP than for cAMP and a higher V_{max} for cAMP than for cGMP. CaM-stimulated PDE (PDE1) has been extensively studied from mammalian tissues (3-9). In most cases, the activation of PDE1 is initiated by binding of Ca^{2+} to free CaM to convert the protein from an inactive state to an active conformation (Ca^{2+}-CaM^*). This activated CaM^* is then associated with the PDE1 to result in the active PDE1 (Ca^{2+}-CaM^*-$PDE1^*$).

Several groups of investigators including ourselves have purified a number of PDE1 from bovine tissues to apparent homogeneity which have a different molecular weights and different subunit structure as well as distinct catalytic and regulatory properties (3-9). It was initially thought that a single form of PDE1 existed in a variety of tissues (10). However, Sharma *et al* (11) provided the first direct evidence for the existence of different isozymic forms of bovine tissue

Table I. Kinetic properties of PDE1 isozymes from bovine tissues.

Isozyme	K_m (μM)		V_{max} (μmol/min/mg)	
	cAMP	cGMP	cAMP	cGMP
PDE1B1	12.0	1.2	10.0	30.0
PDE1A2	35.0	2.7	166.0	93.0
PDE1A1	40.0	3.2	133.0	44.0
Lung PDE1	42.0	2.8	82.0	47.0

For details, see ref. 15.

PDE1, and initially these isozymes were designated according to tissue origin and subunit molecular mass. Since the list of PDE1 isozymes is increasing, a new nomenclature of these isozymes has been suggested by using molecular biology studies, e.g. PDE1B1 (brain 63 kDa PDE1), PDE1A2 (brain 60 kDa PDE1), PDE1A1 (59 kDa heart PDE1) and PDE1C (70 kDa PDE1) (12). At least eleven gene families of PDEs are known to exist (13).

These PDE1 isozymes show subtle differences in kinetic, regulatory properties by CaM, Ca^{2+}, phosphorylation/dephosphorylation and differential inhibition by various pharmacological agents. Furthermore, a potentially novel form of PDE1 regulation by proteolysis and their role in ischemia and reperfusion injury has been observed. The purpose of this review is to summarize some of the most significant advances which have been carried out in our laboratory.

2. Kinetic properties of various PDE1 isozymes

In order to elucidate the physiological significance of the PDE1 isozymes, the purified isozymes were characterized in terms of catalytic properties. Table I shows that PDE1B1, PDE1A2, PDE1A1 and lung PDE1 isozymes have a higher affinity towards cGMP than cAMP. In addition, PDE1A1, PDE1A2 and lung PDE1 isozymes have very similar K_m value whereas the PDE1B1 has a 2-3 fold higher affinity for both substrates (cAMP and cGMP) and a higher V_{max} for cGMP than for cAMP. These results indicate that the PDE1B1 isozyme is kinetically distinct from the other three PDE1 isozymes.

3. Differential regulation of PDE1 by CaM and Ca^{2+}

Although PDE1A1, PDE1A2, lung PDE1 and eye PDE1 isozymes are almost identical both kinetically and immunologically, they are differentially regulated by CaM and Ca^{2+} (14-18). The interaction of Ca^{2+}/CaM and PDE1 in a CaM-stimulated reaction is the inter-dependence of Ca^{2+} binding to CaM and the association of CaM and the enzyme. Various laboratories reported that PDE1A1 had a markedly higher CaM affinity than PDE1B1 and PDE1A2 isozymes (14-18). However, PDE1B1 and PDE1A2 had similar affinity for CaM, but ~10-20-fold lower than that of the PDE1A1 isozymes (15,16). The difference in CaM affinities of isozymes from different tissues has been suggested to represent a mechanism whereby the isozymes accommodate intracellular CaM concentrations of the respective tissues. However, the lung PDE1 isozyme, which contains CaM as a subunit, may be considered the extreme case of a higher affinity CaM binding (19). We have observed that at an identical CaM concentration, PDE1A1 and eye PDE1 isozymes are stimulated at much lower Ca^{2+} concentration than is the PDE1B1 and PDE1A2 (Table II). These results may suggest that differential Ca^{2+} affinity of the tissue specific isozymes may be a mechanism by which the CaM-regulatory reactions are adapted in the respective tissues.

4. Coupling between Ca^{2+} and cAMP second messengers in the regulation of PDE1

The interaction of Ca^{2+} and cAMP second messenger's cascades has been reviewed in detail (3). These two second messengers can interact at multiple sites on at least two levels. On the metabolic level, both cAMP metabolism and Ca^{2+} transport are regulated by both messengers. On the functional level, cellular processes regulated by one messenger can often be modulated by the other. The high degree of complexity in this interactive system could provide the cell with the flexibility required for producing a wide range of specific response reactions.

The activity of PDE1 isozymes can be regulated by several different mechanisms involving Ca^{2+} and cAMP; 1) phosphorylated and non-phosphorylated PDE1 isozymes require Ca^{2+} and CaM for full activity; 2) cAMP or Ca^{2+}/CaM can stimulate PDE1 isozymes phosphorylation; 3) in case of the PDE1A2, PDE1A1 and eye PDE1 Ca^{2+}/CaM can block phosphorylation, whereas Ca^{2+}/CaM stimulate phosphorylation of PDE1B1 and; 4) Ca^{2+}/CaM can reverse phosphorylation of PDE1 isozymes by CaM-dependent protein phosphatase (calcineurin). The multiple regulatory activities of each of the isozymes have to interact under certain defined conditions and their regulatory conditions are summarized in Table III. While PDE1A1, PDE1A2 and eye PDE isozymes are substrates of the cAMP-dependent protein kinase (20-22), the PDE1B1 is phosphorylated by CaM-dependent protein kinase II in a Ca^{2+}/CaM-dependent manner (23). The phosphorylation of PDE1 results in a decrease in the affinity of the isozymes for CaM and an accompanying increase in the Ca^{2+} concentrations required for the isozymes activation by CaM (20-23).

For each of the PDE1 isozymes, a working hypothesis has been postulated to indicate how the multiple regulatory reactions may be organized in the cells to control cAMP concentrations (3-5,8,9). Because these isozymes show low cAMP affinities and low basal PDE1 activities, a major assumption of this working hypothesis is that the isozymes function mainly during cell activations when both cAMP and Ca^{2+} concentrations are elevated. For the regulation of the PDE1A2, PDE1A1 and eye PDE1 isozymes, an increase in intracellular cAMP concentration at the onset of cell stimulation results in the activation of cAMP-dependent protein kinase; the phosphorylation of these isozymes may result in concomitant isozyme inhibition. This will ensure an unimpeded further increase in cAMP concentration. As cellular Ca^{2+} concentration is increased, the CaM-dependent

Table II. Ca^{2+} activation of PDE1 isozymes at various concentrations of CaM.

CaM (μM)	[Ca^{2+}] (μM) Required for half-maximal activation				
	PDE1A1 isoenzyme	PDE1A2 isoenzyme	PDE1B1 isoenzyme	Lung PDE1 isoenzyme	Eye PDE1 isoenzyme
0.1	0.71	ND	ND	ND	0.73
0.5	0.36	ND	ND	ND	0.35
1.00	0.08	0.90	0.70	0.15	ND
10.0	0.01	0.35	0.30	0.15	ND

ND, not determined. For details, see refs. 14,15.

Table III. Regulation of PDE1 isozymes by Ca^{2+} and cAMP.

Isozyme	Regulation
Brain PDE1A2 Heart PDE1A1 Eye PDE1 isozyme	1. Activation by Ca^{2+} and CaM. 2. Phosyphorylation by cAMP-dependent protein kinase to result in an increase in the Ca^{2+} concentration required for phosphodiesterase activation. 3. Blockage of phosphodiesterase phosphorylation by Ca^{2+} and CaM. 4. Reversal of the phosphorylation by CaM-dependent phosphatase.
Brain PDE1B1	1. Activation by Ca^{2+} and CaM. 2. Phosyphorylation by CaM-dependent protein kinase II to result in an increase in the Ca^{2+} concentration required for phosphodiesterase activation. 3. Reversal of the phosphorylation by CaM-dependent phosphatase.

For details, see refs. 19-24.

protein phosphatase is activated to reverse the phosphorylation of PDE1A2, PDE1A1 and eye PDE1, and the dephosphorylated isozymes are then activated by Ca^{2+} and CaM. Because the association of CaM to these isozymes prevents the phosphorylation of the isozymes by the protein kinase (Table III), the isozymes will remain in its dephosphorylated state even if cAMP concentration remains high. The concerted actions of Ca^{2+} and CaM on the phosphatase reaction, the phosphorylation reaction, and PDE1 isozymes activity lead to a decline in cAMP concentration in the cells.

The multiple regulatory reactions for PDE1B1 all involve Ca^{2+} and CaM (Table III), and they can affect the PDE1B1 activity in opposite directions. The CaM-stimulation of the isozyme is partly opposed by the CaM-dependent isozyme phosphorylation, which, in turn, is counteracted by the CaM-stimulated phosphatase reaction. To reconcile such opposing effects by Ca^{2+}/CaM, it is postulated that the distinct CaM-dependent regulatory reactions are temporally separated during the cell stimulation; that is, the protein kinases, which act in concert with adenylate cyclase to increase cAMP concentrations, are activated by CaM prior to the activation of the phosphatase and the PDE1B1. Activation of PDE results in a decline in intracellular cAMP concentrations.

5. Role of autophosphorylation of PDE1B1 by CaM-dependent protein kinase II

Like other protein kinase II isozymes, bovine brain CaM-stimulated protein kinase II converts from a CaM-dependent to a CaM-independent active protein kinase upon autophosphorylation. The desensitization of the autophosphorylation reaction to Ca^{2+} and CaM occurs within a few seconds. Both autophosphorylation and the phosphorylation of PDE1B1 by the CaM-dependent protein kinase II are independent of Ca^{2+} within 15 sec (24). Thus, one possible mechanism wherein a temporal separation of PDE1B1 phosphorylation from CaM-stimulations of PDE1B1 dephosphorylation and PDE1B1 activity is achieved may be through the CaM-dependent protein kinase II autophosphorylation reaction. A more detailed description of bovine brain CaM-stimulated protein kinase II and discussion of the role of its autophosphorylation in the regulation of PDE1B1 have been reviewed (3-5,8,9).

6. Differential inhibition of PDE1 isozymes and its therapeutic applications

PDEs are involved in maintaining (attenuating) the dynamic changes in the levels of cAMP and cGMP. In most cells and

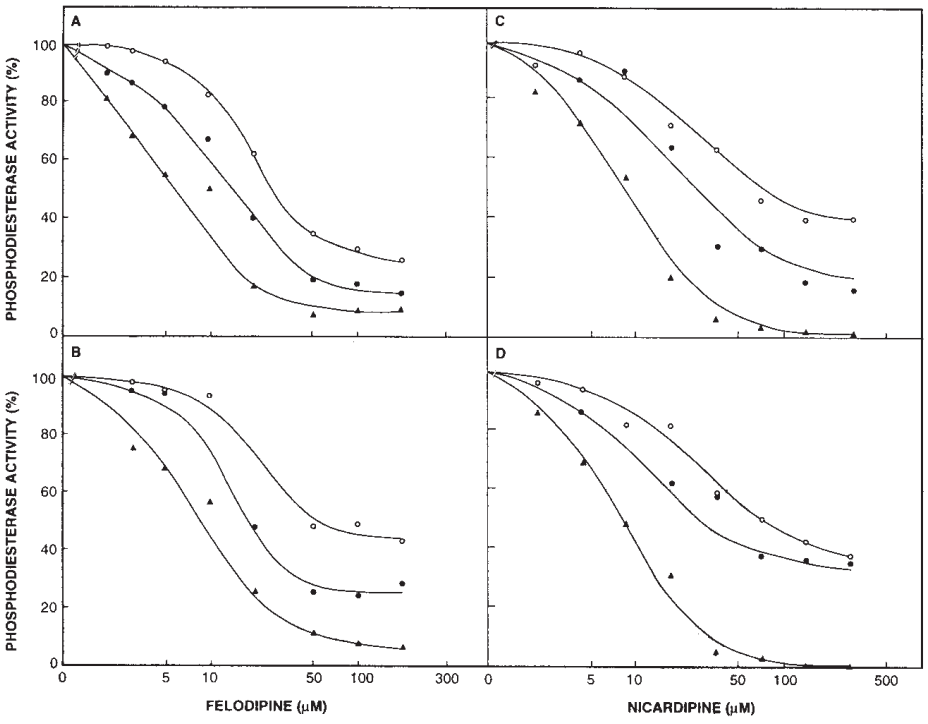

Figure 1. Effect of dihdropyridine Ca^{2+} antagonists on bovine brain PDE1 isozymes in the presence of Ca^{2+} and CaM. PDE1 activity was measured in the presence of three different concentrations of cAMP (40 μM, ▲-▲; 400 μM, ●-●; 1.2 mM, ○-○). PDE1A2 isozyme, A and C; PDE1B1 isozyme, B and D along with varied concentrations of felodipine (A and B) and nicardipine (C and D). For details of experimental conditions, see Sharma *et al* (38).

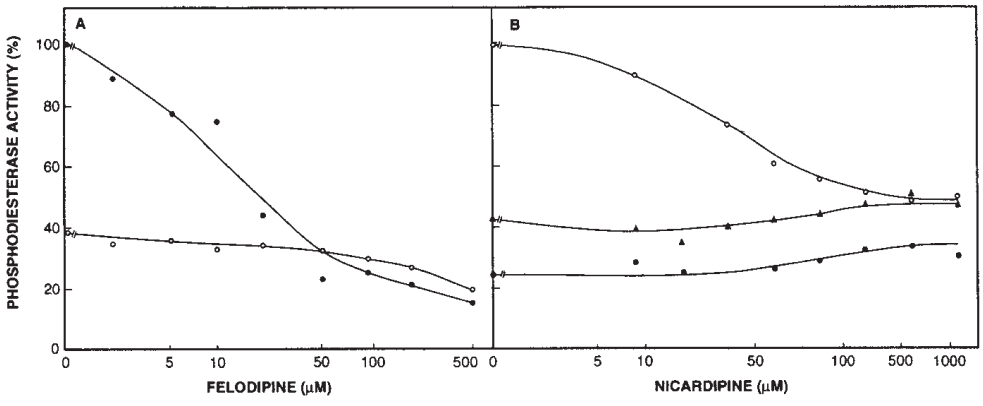

Figure 2. Specific binding site for dihydropyridine Ca^{2+} antagonists on bovine brain PDE1 isozymes. A, inhibition of PDE1A2 isozyme by various concentrations of felodipine alone (●-●) and in the presence of 1.12 mM nicardipine (○-○). B, inhibition of PDE1A2 isozyme by various concentrations of nicardipine alone (○-○) and in the presence of felodipine (50 μM, ▲-▲; 100 μM, ●-●). The enzyme activity was determined at 1.2 mM cAMP in the presence of Ca^{2+} and CaM. For details of experimental conditions, see Sharma *et al* (38).

tissues, the capacity for hydrolysis of cyclic nucleotides by PDEs is >10 times the maximum rate of synthesis of cAMP and cGMP (13). Therefore, a partial inhibition of PDEs would result in many-fold increase in the level of cyclic nucleotides in the cell and thus the activation of cAMP-dependent protein kinase (25,26). The PDE activity needs to be tightly regulated for proper functioning of the signaling pathways, which are linked to many cellular processes. Selective inhibitors are being discovered and used to regulate the PDE activities during normal pathological conditions.

A variety of pharmacological agents have been used to inhibit PDE1 isozymes, and this occurs mostly via Ca^{2+}-dependent association with the proteins. Furthermore, in previous studies it was unclear which of the specific PDE1 isozymes were used. Moreover, these studies were carried out by using partially purified PDE1. We have demonstrated that PDE1 isozymes may be differentially inhibited by several pharmacological agents (Table IV).

Earlier studies have demonstrated that nicardipine does not have any significant differences in the inhibition of PDE1 isozymes (27); however, isobutylmethylxanthine (IBMX) exhibits marked differences in its inhibition potency for the PDE1A1, PDE1A2, and PDE1B1 isozymes (27). Ginsenosides are used for treatment of heart failure and to protect tissue from

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

Isozyme	Pharmacological agents						
	I BMX (μM)	Nicardipine (μM)	Ginsenoides			Deprenyl (μM)	Amantadine (μM)
			Rc (μM)	Rb (μM)	Re (μM)		
Heart PDE1A1	6.0	4.0	3.7	6.8	14.8	NC	ND
Brain PDE1A2	7.0	1.4	3.7	6.3	12.7	1.0	4.8
Brain PDE1B1	36.0	2.0	ND	ND	ND	2.0	ND

NC, not carried out; ND, not detected. For details, see refs. 27,30-32.

damage when an organism is under stress (28,29). Therefore, the effects of various ginsenosides were examined on bovine brain and heart PDE1 isozymes (30). We have demonstrated that ginsenosides were found to be potent inhibitors of PDE1A1 and PDE1A2 isozyme but not of PDE1B1 isozyme (30). Deprenyl (selegeline hydrochloride), which is a selective inhibitor of monoamine oxidase-B, inhibits PDE1A2 isozyme but is a poor inhibitor for PDE1B1 isozyme (31). In addition, amantadine is used for the treatment of Parkinson's disease and is also a potent drug for other disorders such as stroke, epilepsy, and neuroleptic malignant syndrome. It is interesting to note that amantadine only inhibits PDE1A2 isozymes but not the PDE1B1 and PDE1A1 isozymes (32). Since the inhibition of PDE1 isozymes by these compounds was overcome by increasing the concentration of CaM, this suggests that these compounds act specifically and reversibly against the action of the CaM (30-32). Therefore, these compounds should be valuable tools to investigate the diverse physiological roles of distinct PDE1.

Some dihydropyridine Ca^{2+} antagonists show CaM antagonist activity and inhibit multiple PDE1 isozymes (33-37). However, it was not clear which of the PDE1 isozymes were used (33-37). Therefore, the effects of dihydrophyridine Ca^{2+} antagonists were examined on PDE1A2 and PDE1B1 isozymes at various concentrations of felodipine and nicardipines (38). The dose-dependence curve in the presence of Ca^{2+} and CaM at various concentrations of cAMP shows the effects of felodipine (Fig. 1A and B) and nicardipine (Fig. 1C and D) on the PDE1A2 and PDE1B1 isozymes, respectively. These results suggest that felodipine and nicardipine appear to be only partially competitive inhibitors because inhibition of PDE1 isozymes by felodipine and nicardipine does not approach 100% at high concentration of substrate (cAMP). In addition, this suggests that dihydropyridine Ca^{2+}-antagonist binds to an enzyme site distinct from the active site. To further substantiate that PDE1 isozymes contain specific dihydropyridine Ca^{2+}-antagonist binding sites distinct from the enzyme-active site, the inhibition of PDE1A2 isozyme was carried out by various concentrations of felodipine and in the presence of nicardipine (Fig. 2A) or by various concentrations of nicardipine and in the presence of felodipine (Fig. 2B). The results suggest that both nicardipine and felodipine bind at a specific site and, therefore, counteract each other's effect on the enzyme activity. This study is the first to substantiate the existence of specific sites, distinct from the active site on PDE1, that show high-affinity binding of these drugs. Felodipine and nicardipine have similar affinities for the PDE1A2 isozyme but bring about different levels of enzyme inhibition. This suggests the possibility of designing specific drugs that protect the enzyme from inhibition by such Ca^{2+} antagonists. As such, the possible molecular mechanisms of the interaction between drugs and intracellular targets require further study.

7. Role of proteolysis in regulating PDE1A2

Sequences containing several regions rich in proline (P), glutamic acid (E), serine (S) and threonine (T) are known as 'PEST' regions. The PEST regions have been suggested to be recognized by specific proteases, particularly the Ca^{2+}-dependent cysteine proteases, calpains (39,40). Calpains are one of the major mediators for the Ca^{2+} signal in many biological systems and they are ubiquitously expressed in all tissues (41,42).

We have shown that PDE1 is proteolyzed by *m*-calpain (43). Proteolytic cleavage of PDE1A2 by *m*-calpain generated a 45 kDa immunoreactive fragment, which is an active CaM-independent form (Fig. 3, inset). The time course of conversion of PDE1A2 into a CaM-independent form is shown in Fig. 3. 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 (Table V). High molecular weight CaM-binding protein (HMWCaMBP), which was discovered in our laboratory (44), is homologous to capastatin (45) an endogenous inhibitor of calpains and did not alter PDE1A2 activity but did inhibit calpain-mediated proteolysis (Table V). A CaM overlay using biotinylated CaM revealed that the 45 kDa fragment does not contain the CaM binding domain (43). 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 *m*-calpain action. A previous study on connexin-32 indicated that its phosphorylation by protein kinase C prevents proteo-

Table V. Effect of *m*-calpain on PDE1A2 activity.

	PDE1A2 activity[a]	
	Ca^{2+}/CaM	EGTA
PDE1A2	42.7±1.0	4.2±0.2
m-calpain	1.0±0.0	1.0±0.0
PDE1A2 + EGTA + *m*-calpain	45.0±1.9	3.8±0.2
PDE1A2 + Ca^{2+} + *m*-calpain	48.8±1.1	47.5±1.0
PDE1A2 + Ca^{2+} + CaM + *m*-calpain	46.6±1.0	44.2±1.4
PDE1A2 + calpastatin	42.1±1.6	5.2±0.6
PDE1A2 + Ca^{2+} + calpastatin + *m*-calpain	41.0±1.0	4.1±0.3

For details, see ref. 43. [a]One unit of enzyme activity is defined as μmol of cAMP hydrolyzed/min at 30˚C.

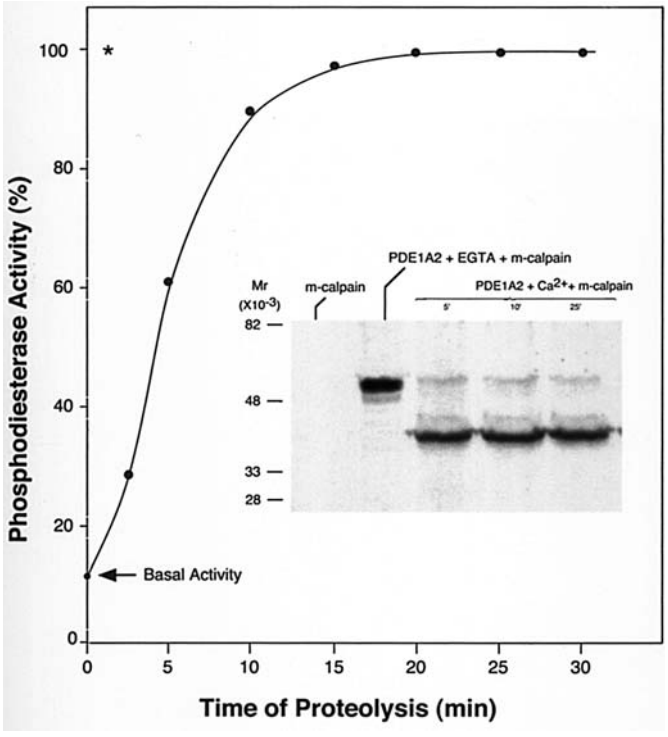

Figure 3. Effect of proteolysis on PDE1A2 activity. PDE1A2 was incubated with *m*-calpain in the presence of Ca^{2+}, and at the times indicated proteolytic reaction was stopped with leupeptin and aliquots were assayed for activity in the presence of 5 mM EGTA. The arrow indicates the basal enzyme activity, 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%. Inset: Western blot analysis of PDE1A2 after incubation with *m*-calpain in the absence or presence of Ca^{2+} (43).

lysis by calpains (46). It has been shown that two serine residues of PDE1A2 are phosphorylated by cAMP-dependent protein kinase, at serine 112 and serine 120 (47). Our results suggested that the calpain cleavage site could be distant from the phosphorylation site (43). The cleavage site for calpains rarely resides in the PEST motif (48), although in some calpain substrates, PEST sequences are located near the cleavage site. 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 (43).

The domain organization of the PDE1A2 revealed that the native protein is a 530-residue polypeptide, a homodimer that is N^{α}-acetylated, and is composed of separate catalytic and regulatory domains (49,50). From the N-terminus, the CaM binding site is located from residue 23 to 41, the PEST motif from residue 73 to 94 and the phosphorylation site from residue 110 to 120, respectively. The catalytic domain encompasses an approximately 250-residue sequence (139-446) which is conserved among PDE1 isozymes of diverse size, phylogeny and function (6). Our analysis revealed 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 Glutamine 126 and Alanine 127 (Fig. 4).

The 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 (43). 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 (51-54). It has been reported that in patients having Parkinson's disease with dementia, there is a significant decrease in cAMP (55). Earlier, we have demonstrated that PDE1A2 is inhibited by anti-parkinsonian agents, suggesting a potential role of PDE1 in Parkinson's disease (31,32). Since PDE1A2 is predominantly expressed in brain (6,53,56), 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 it independent of CaM. Since PDE1A2 also has an

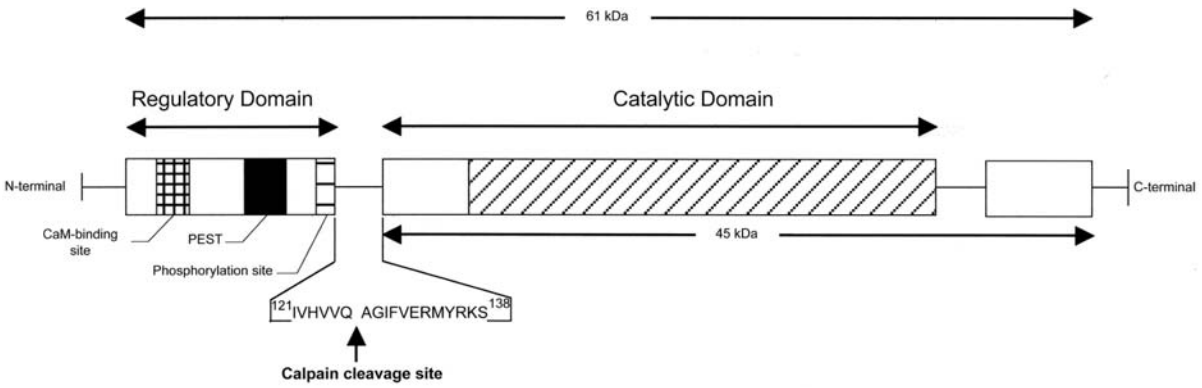

Figure 4. A representation of the PDE1A2 showing the cleavage site by *m*-calpain. The regulatory and catalytic domains are shown. The lined, black, and checkered boxes represent the CaM-binding domain, PEST, and phosphorylation site followed by conserved catalytic domain (hatched area), respectively (43). The arrow indicates the *m*-calpain cleavage site between residues Glutamine 126 (Q) and Alanine 127 (A).

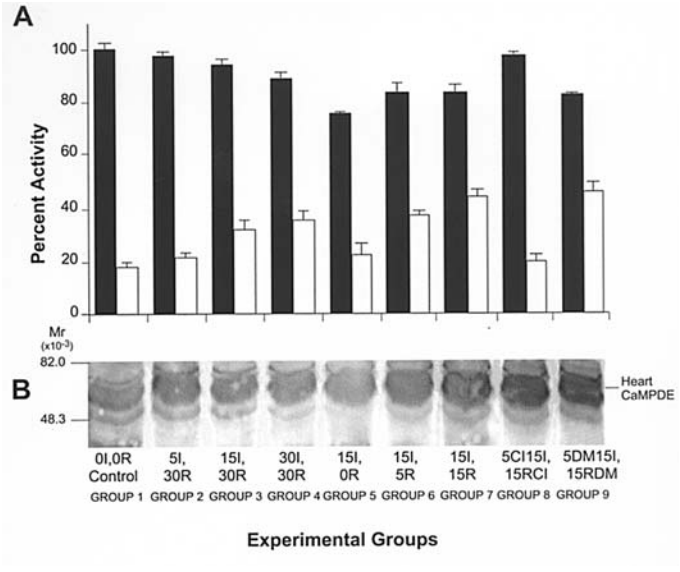

Figure 5. PDE1A1 activity in experimental groups of rat heart ischemia-reperfusion. A, PDE1A1 activity in the presence of Ca^{2+}/CaM (closed bar) or EGTA (open bar). B, Immunoblot of PDE1A1 from normal and ischemia-reperfusion rat heart tissues supernatant. Group 1, control (no ischemia or perfusion); group 2, 5 min ischemia, 30 min reperfusion (5I, 30R); group 3, 15 min ischemia, 30 min reperfusion (15I, 30R); group 4, 30 min ischemia, 30 min reperfusion (30I, 30R); group 5, 15 min ischemia, 0 min reperfusion (15I, 0R); group 6, 15 min ischemia, 5 min reperfusion (15I, 5R); group 7, 15 min ischemia, 15 min reperfusion (15I, 15R); group 8, hearts were perfused with 100 μM calpain inhibitor in 0.1% DMSO for 5 min before 15 min of ischemia and again during 15 min reperfusion (5CI15I, 15RCI) (CI, calpain inhibitor (ALLM); and group 9, with 0.1% DMSO alone as described in group 8 (5DM15I, 15RDM) (DM, dimethyl sulfoxide). Values are mean ± SD of two samples in each group (57).

affinity for cGMP (14), 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 PDE1 by *m*-calpain may provide an important route for controlling the intracellular level of cAMP and the physiological processes regulated by these messenger molecules.

8. Role of PDE1A1 in ischemic-reperfused heart

The role of PDE1A1 in cardiac ischemia remains unknown. Therefore, we examined PDE1A1 activity and expression, cAMP concentration and the effect of a calpain inhibitor in cardiac ischemic-reperfused rat model (57). We demonstrated, for the first time, that ischemia-reperfusion injury to rat myocardium alters the activity of heart PDE1A1 while having minimal effects on cAMP concentrations (57). The total PDE1A1 activity (Fig. 5A) and expression (Fig. 5B) in rat heart was largely unchanged under various conditions of ischemia and reperfusion. However, the basal activity which is independent of Ca^{2+}/CaM, increased with increasing degree of ischemia reperfusion (Fig. 5A). Pretreatment of rat hearts with cell permeable calpain inhibitor (ALLM, N-Ac-Leu-Leu-methioninal) appeared to have a protective effect on PDE1A1 activity as total PDE1A1 activity in this group was found to be 97% of the control group (Fig. 5A). However, protein expression of PDE1A1 was not altered in the ischemic and reperfused rat hearts as indicated (Fig. 5B).

The protective effect of calpain inhibitor upon PDE1A1 in rat heart appears to be due to a decrease in calpain-mediated proteolysis of PDE1A1. *In vitro* incubation of PDE1A1 with μ- and *m*-calpain demonstrated that the PDE1A1 isozyme was susceptible to proteolysis by the calpains (Fig. 6). Furthermore, we have reported that *m*-calpain was capable of generating CaM-independent forms of PDE1A2 proteolysis (43). This study reports the first observed proteolysis of heart PDE1A1 by both μ- and *m*-calpain (57). Such a pathway allows for decoupling of the Ca^{2+} signal from PDE1A1 under sustained conditions of increased intracellular Ca^{2+}.

While PDE1A1 activity varied in our experimental model (Fig. 5A), the total cAMP concentrations in rat myocardium appeared to be only slightly affected (Fig. 7). The generation of a CaM-independent PDE1 during ischemic events may therefore allow for the maintenance of cAMP levels in the face of Ca^{2+} derangements, which follow ischemic events (58). During reperfusion, an increased uptake of Ca^{2+} has been observed in myocytes (59), which would be capable of activating calpains. These activated calpains could then proteolyse PDE1A1 and irreversibly activate it, preventing the potentially toxic accumulation of cAMP in myocytes.

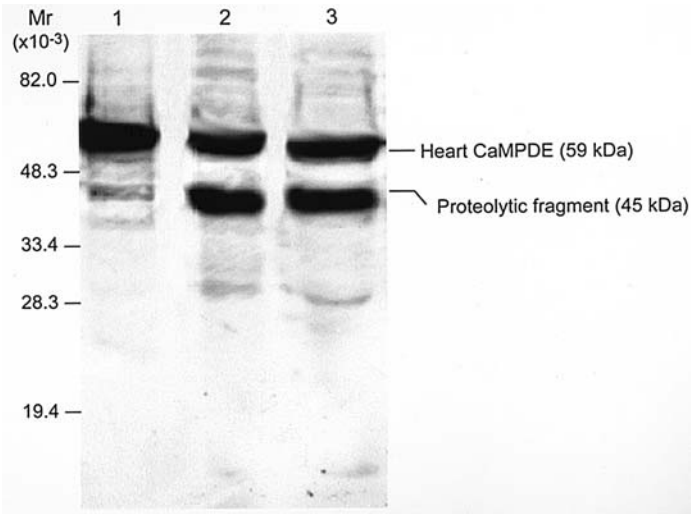

Figure 6. *In vitro* proteolysis of purified bovine cardiac PDE1A1 by μ-calpain and *m*-calpain. Lane 1, bovine heart PDE1A1 alone; lane 2, bovine heart PDE1A1 and μ-calpain; lane 3, bovine heart PDE1A1 and *m*-calpain (57).

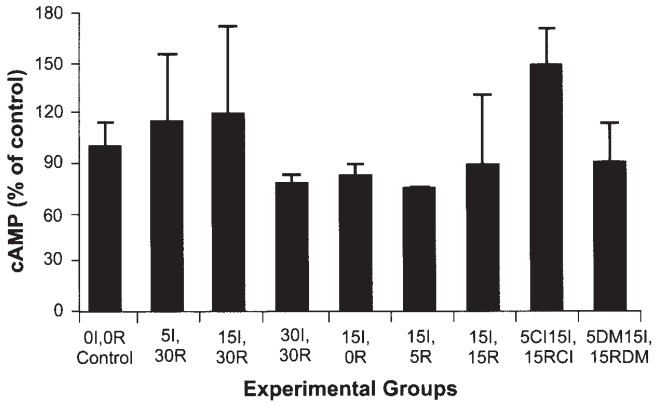

Figure 7. cAMP concentrations in experimental groups of ischemia-reperfusion in heart. Details for various groups, see Fig. 5. Values are mean ± SD of two samples in each group (57).

A previous study from our laboratory indicated that the protein expression of calpains and the endogenous calpain inhibitor HMWCaMBP are altered during cardiac ischemia-reperfusion (60). HMWCaMBP appears to be cardioprotective during periods of ischemia and have decreased expression during reperfusion. We also observed that pretreating ischemic hearts with calpain inhibitor significantly reduced the incidence of ventricular fibrillation (60). However, during prolonged periods of ischemia and reperfusion, a decrease in the calpain inhibitory activity of HMWCaMBP was observed. It appeared that the loss of calpain inhibitory activity was due to proteolysis of HMWCaMBP by calpains, which are activated during periods of ischemia and reperfusion. An increase in the expression of both μ- and *m*-calpain was observed in ischemia-reperfusion hearts as compared with control groups (60). With the increase in calpain activity there was a corresponding loss of HMWCaMBP activity and expression, which allows for further calpain-mediated proteolysis of cardiac tissue. Thus, during ischemia-reperfusion, calpain activity is increased through both an increase in the expression of calpains and a decrease in its endogenous inhibitor, HMWCaMBP.

It has been reported that during ischemia and reperfusion there is an increased influx of Ca^{2+} into the cells, which can activate calpains (61-63). Disturbances in the precise control of intracellular Ca^{2+} can result in the dysregulation of various molecular events and can compromise cell viability (64). Calpains, calpastatin and HMWCaMBP play an important role in various physiological and pathological processes (40,65-72). Calpains are concentrated in the Z disk, the site where myofibril disassembly begins (73). It has been reported that calpains are activated by Ca^{2+}, and the treatment of purified myofibrils with Ca^{2+} causes rapid and complete loss of the Z disk and partial degradation of M lines (74). One of the physiological functions of Z disks and M lines is to maintain the architecture of myofibrils, and their disintegration can lead to masses of disorganized filaments (74,75). Calpains also degrade tropomyosin and C protein, which contribute to the stability of thin and thick filaments, respectively (76,77). Other cardiac contractile proteins including desmin, troponin (T, C and I), filaments, nebulin, gelsolin, titin, α-actinin, and myosin also act as substrates of calpains (77-82). Their degradation can result in the contractile derangements and disassembly of sarcomere proteins caused by myocardial ischemia and could also be important for other pathological conditions (77,82). Therefore, calpains have a unique specificity for degradation of those structural proteins that serve to keep actin and myosin assembled in the form of myofibrils. Calpains are also widely distributed in myocytes and are implicated in myocardial stunning and ischemia reperfusion injury (61,62,66). Increased disruption of microtubules is associated with its action, by increasing intracellular Ca^{2+} (63).

While cAMP is necessary for the normal and effective cardiac function (83,84), it has also been shown to have arrythmogenic effects, which can be demonstrated by the use of PDE inhibitors (28). A loss of cardiac response to ß-adrenergic receptor stimulation, which increases cAMP, has also been documented in cardiac tissue under ischemic and cardiac failure conditions (85,86). By preserving the degradative portion of the cAMP pathway via generation of CaM-independent PDE, a physiologic mechanism of maintaining cAMP in acute and chronic myocardial injury exists, thereby preventing further myocardial damage. Thus, a dynamic interaction between calpains and PDE1A1 in an ischemia-reperfusion model of the heart may allow for the maintenance of physiological concentrations of cAMP during periods of hypoxic injury to the heart. This definitely merits further in-depth studies.

9. Conclusion

PDEs are the enzymes which terminate the action of cAMP and cGMP by hydrolyzing them to their respective 5'-nucleotide monophosphates. One of the most intensively studied PDEs is the PDE1 which is involved in the complex interactions between the cAMP and Ca^{2+} second messenger systems. Immunological, kinetic, activation and regulatory characterizations have revealed subtle differences between PDE1 isozymes. In addition, our *in vitro* studies suggest that

the activity of PDE1 is selectively regulated by cross-talk between Ca^{2+} and cAMP signaling pathway. The different PDE1 isozymes can be distinguished by various pharmacological agents. This study leads to the possibility of developing PDE1 isozyme-specific inhibitors which may be therapeutically useful. Furthermore, we have shown that PDE1 is proteolyzed by a Ca^{2+}-dependent cysteine protease, calpains. This study suggests that limited proteolysis by calpains could be an alternative mechanism for activation of PDE1. In addition, we have demonstrated PDE1A1 activity expression and effect of calpains in the rat model *in vitro* of cardiac ischemia-reperfusion. The physiological functions of PDE1 isozymes are still not clear since most studies undertaken so far have been on *in vitro* systems; therefore, it is essential that further research be directed to *in vivo* studies.

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