Chapter

Role of cAMP Homeostasis in Intra-Macrophage Survival and Infectivity of Unicellular Parasites like *Leishmania*

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Abstract

Unicellular eukaryotic pathogen *Leishmania donovani*, an intra-macrophage protozoan parasite, on exposure to phagolysosome conditions (PC) of mammalian macrophages, show increased cAMP level and cAMP-dependent protein kinase A (PKA) resulting in resistance to macrophage oxidative burst. In order to have a comprehensive understanding of cAMP signaling and their contribution to infectivity, studies were carried out on all the enzymes associated with cAMP metabolism such as adenylate cyclase, phosphodiesterase, pyrophosphatase and the regulatory and catalytic subunits of PKA. This chapter deals in detail the contribution of these components of cAMP signaling in cAMP homeostasis of the parasite as well as their role on successful host-parasite interaction leading to intracellular parasite survival and establishment of infection. Finally, a discussion is made about how these observations might be exploited for developing drug candidates targeting parasite specific features.

Keywords: *Leishmania*, parasite, cAMP, phosphodiesterase, pyrophosphatase, receptor adenylate cyclase, infectivity

1. Introduction

Leishmaniasis, caused by protozoan parasite *Leishmania* is still endemic in many countries and is considered as one of the potent neglected tropical disease. There are three main forms of leishmaniases-visceral (also known as kala-azar and the most serious form of the disease), cutaneous (the most common), and mucocutaneous form of the disease. Though there are surveillance and control measures for leishmaniasis being used by the World Health Organization, the treatment regime of the disease is yet to be enough to eradicate the disease worldwide. There are continuous research on potential new treatments and possible vaccines for leishmaniasis, but adequate treatment is still unavailable.

Unicellular eukaryotic pathogen *Leishmania donovani*, when exposed to phagolysosome conditions (PC) of macrophages (37°C and pH 5.5); a pre-requisite for parasite survival and infectivity, showed to elevate cAMP level and cAMP-mediated protein kinase A (PKA) activation. In eukaryotes, several researches indicate that most of the cAMP mediated effects are due to the activation of the cAMP-regulated protein kinase A, and the subsequent phosphorylation of other substrates of PKA

which act as transcription factors, or metabolic enzymes such as lipases, phosphorylase kinase or glycogen synthase. In unicellular eukaryotes, there are many reports which implicates cAMP as one of the major environmental sensing machineries associated with stress response in *Plasmodium*, *Trypanosoma*, *Toxoplasma* and others. In malarial parasite, *Plasmodium falciparum*, cAMP is one of the main molecules responsible for the formation of sexual precursor, gametocytes from the asexual forms [1]. *P. falciparum* produces its own cAMP requirement by receptor adenylate cyclase (AC) which seemed to be unaffected by the well-known mammalian RAC activator Forskolin or heteromeric G-protein activators fluoroaluminate ($A1F_4^-$). Moreover, cAMP signaling effector molecule protein kinase A (PKA) plays an important role in conductance of anions across the host cell membrane of *Plasmodium*-infected RBC [2]. Moreover, recent researches showed that PKAR (PKA regulatory subunit) is functionally associated with the activation of anion conductance channel in *P. falciparum*-infected RBC [3]. cAMP-dependent signaling pathway activation and PKC activation in *Entamoeba histolytica* triggers the phosphorylation of proteins involved in actin rear-arrangements necessary for its movement and adhesion. Moreover, cAMP-response elements could play an important role in regulating actin expression and organization in signaling processes activated during tissue invasion. However, there are several other reports of mechanisms of cAMP action, such as the direct regulation of ion channels in olfactory cells, or the activation of chemotactic receptors in the slime mould, *Dictyostelium*. In unicellular eukaryotes like *Toxoplasma gondii*, both cyclic GMP (cGMP) and cyclic AMP (cAMP) can induce bradyzoite formation. These effects could be due to an increase in host or parasite cyclic nucleotides. Host cell environments including cAMP elevations contribute to the bradyzoite differentiation process in *T. gondii*, which has a receptor or sensor for cyclic nucleotides [4]. In *Dictyostelium*, cAMP secreted into the environment binds to cAMP receptors to regulate the differentiation program of cells within the fruiting body [5]. In *Leishmania*, the mechanism of action of cAMP signaling represent a particularly intriguing question since the major pathway of cAMP signaling in eukaryotes, the regulation of transcription, does not seem to be applicable because kinetoplastid parasites like *Trypanosoma* and *Leishmania* exhibit obscure transcriptional regulation. An attempt to understand cAMP signaling in *Leishmania* was undertaken by Seebeck and his group and initial studies in *L. major* where they identified five PDE genes, PDEA, PDEB1, PDEB2, PDEC and PDED encoding class I enzymes similar to those found in higher eukaryotes [6].

The protozoan parasite *Leishmania donovani*, when exposed to stress condition in the mammalian macrophages, encounter an oxidative burst as the first line of defense, offered by the macrophages by producing reactive oxygen species and reactive nitrogen intermediates [7, 8]. Still, a subset of the parasites can survive and transforms into amastigotes leading to disease manifestation [9, 10]. In *Leishmania*, cAMP is one of the major players driving the transformation of the parasite from promastigotes to amastigotes and allowing survival of parasites in macrophages [11]. Not only in the differentiation of *Leishmania*, cAMP also an important role in the differentiation of *Trypanosoma* from slender form to short stumpy form [11]. In kinetoplastid parasite *Trypanosoma*, cAMP levels are modulated all through the different stages of the cell cycle plays a significant function in transformation from slender forms to stumpy forms [12]. Also a stumpy induction factor (SIF) has been reported in *Trypanosoma* which triggers cell cycle arrest in G_1/G_0 phase and induces differentiation with high efficiency and elicits an immediate two- to three-fold elevation of intracellular cAMP content upon addition to slender forms [13]. Membrane-permeable derivatives of cAMP or the phosphodiesterase inhibitor etazolate perfectly mimic SIF activity in *Trypanosoma*. Moreover, it was also shown that the transformation in *Trypanosoma* was not mediated directly by cAMP

or cAMP-analogs but by the products of hydrolysis of the membrane permeable cAMP-analogs [14]. In *Leishmania*, previous studies also showed that cAMP causes G_1 arrest in cell cycle which perhaps aids the parasite transformation [15]. Although cAMP seemed to induce cell cycle arrest in *Leishmania*, little is known about the intricate mechanism of the arrest. Though spatiotemporal regulation of cAMP and slight changes of it seemed important in the parasite, scanty data exist regarding the potential toxicity of *Leishmania* cells to pharmacologic elevation of cAMP levels. Moreover, in several mammalian systems, elevation of cAMP level is one of the stimuli that can induce growth arrest or cell death (or both) in many cultured lymphoid cells, including resting B cells, germinal center B cells, T lymphocytes, and thymocytes [16–20]. cAMP also induces cell death in cells derived from lymphoid malignancies, including murine lymphoma cell line S49.1, B-CLL cells, and multiple myeloma cells [21, 22].

To understand the importance of canonical cAMP signaling components, enzymes associated with cAMP metabolism were studied. cAMP is universally generated by adenylate cyclase in a G-protein coupled receptor signaling cascade, which catalyzes the cyclization of ATP to cAMP. In *Leishmania*, the absence of G-proteins made this signaling cascade a unique one. In many instances, adenylate cyclase is regulated by various molecules including bicarbonate, calcium, and hormones. Interestingly, our studies confirmed the importance of inorganic pyrophosphate pool (PPi), an energy storage compound and byproduct of cAMP synthesis, as one of the regulators of receptor adenylate cyclases in *Leishmania*. Also, amongst the stage specific receptor adenylate cyclases, LdRAC-A showed to regulate cAMP levels in the parasite when exposed to phagolysosome conditions. The PPi pool seemed to a stringent control by membrane bound pyrophosphatases of acidocalcisomes (ACms). Downstream, a differentially expressed soluble cytosolic cAMP phosphodiesterase (LdPDEA) and another cytosolic cAMP-dependent PDE, LdPDED, seemed responsible for controlling cAMP homeostasis. Also, a functional cAMP-binding effector molecule from *L. donovani* (a regulatory subunit of PKA, LdPKAR) seemed important in parasite infectivity playing a substantial role in autophagy induction, an event important for parasite transformation in phagolysosome conditions. Protein phosphorylation in a cAMP-dependent manner is important in the life cycle of the parasite and in any trypanosomatids, the pattern of protein phosphorylation changes within the life cycle of the parasite [23–32].

This chapter will deal in detail, the components of cAMP signaling in the parasite and unequivocally demonstrate their contribution in cAMP homeostasis; an important event for parasite survival, successful host-parasite interaction, which might be exploited for developing drug candidates targeting parasite specific features.

2. cAMP and associated enzymes in *Leishmania*

In eukaryotes, cAMP a second messenger, is an essential molecule playing a vital role in intracellular signaling which control a vast array of cellular events like cytoskeletal modeling, proliferation, virulence, differentiation and apoptosis [33]. cAMP is formed from adenosine triphosphate (ATP) by receptor adenylate cyclases (RAC). In *Leishmania*, there are reports of several isoforms of both membrane bound receptor adenylate cyclases [34] as well as soluble adenylate cyclases. When cAMP is produced, inorganic phosphate (Pi) is also produced as one of by-product of the reaction. Regulation of the pyrophosphate (PPi) pool formed by the accumulation of Pi, is hydrolysed by pyrophosphatase. In *Leishmania*, there are three isoforms of pyrophosphatases: Inorganic pyrophosphatase (IoPPase), vacuolar proton transporting pyrophosphatase ($V\text{-}H^+PPase$) and acidocalcisomal soluble

pyrophosphatase (VSP1). Downstream to cAMP, leishmanial phosphodiesterases (PDE) hydrolyzes cAMP to 5′ adenosine monophosphate (5′AMP). There are five different PDEs in the parasite (PDEA, PDEB1, PDEB2, PDEC, and PDED). cAMP-dependent protein kinase A (PKA) exists as an inactive tetramer consisting of two catalytic subunits (PKAC) and two regulatory subunits (PKAR). Binding of cAMP to PKAR releases PKAC subunit.

2.1 Receptor adenylate cyclase in *Leishmania*

cAMP signaling cascade is activated only when local cAMP concentration reaches a level high enough to activate a cAMP responsive respective effector protein/s. It has been observed that mostly, the activation threshold lies around 1 ± 10 mm. The increase of cAMP from a basal level can be brought about either by the activation of one or several RACs, or by the inactivation of the PDEs. In eukaryotic cells, cAMP is predominantly generated at the plasma membrane since most of the known RACs are integral membrane proteins. From the site of its generation, the cAMP diffuses until it hits the respective effector molecule, or until it is hydrolysed by PDEs (**Figure 1**). The cAMP signal can take the form of a diffusion-controlled concentration gradient [35], it can be delivered in the form of time- and space-controlled spikes of cAMP concentration or consists of a sustained increase or decrease in intracellular cAMP concentration. Adenylate cyclase-cAMP pathway is also involved in the internalization process of the parasite by the host cells [36].

Studies have confirmed that cAMP is involved in signal transduction events occurring during transformations in *Leishmania* and other related kinetoplastid protozoa. Different life cycle stages contain different intracellular concentrations of cAMP in *Trypanosome brucei* [37] and in *T. cruzi* [23]. Furthermore, cAMP analogs and phosphodiesterase inhibitors promote *in vitro* differentiation of non-infectious epimastigotes of *T. cruzi* into infectious metacyclic trypomastigotes [38]. This major

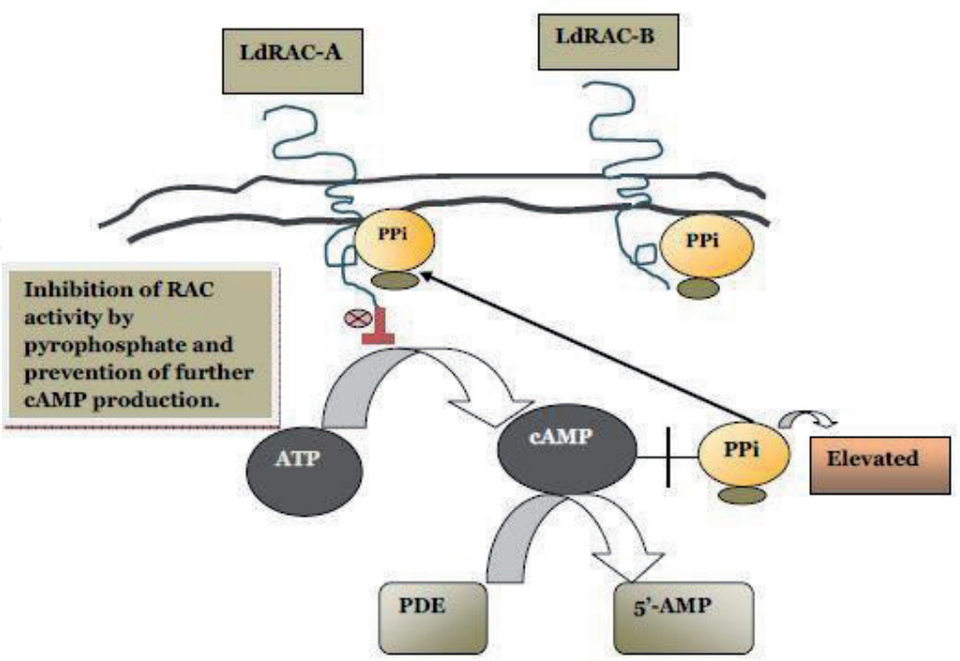

Figure 1.
Receptor adenylate cyclase in Leishmania*: PPi inhibiting adenylate cyclase. In normal condition, when the parasites are not exposed to stress, receptor adenylate cyclase synthesizes cAMP from ATP and PPi is produced as by-product. This PPi interacts with the receptors and inhibits further synthesis of cAMP. On the other hand, PDEs present in the cell also helps in maintaining the concentration of cAMP by hydrolyzing cAMP to 5′-AMP. So, both PPi produced and the PDEs help in the regulation of cAMP level in the parasite.*

role of cAMP in the transformation of kinetoplastid protozoa has led to the investigation of adenylate cyclases in *L. donovani*. A family of five clustered genes in *L. donovani* was identified which encodes signal transduction receptors [39]. The coding region of these genes was sequenced and have been shown to code for proteins with an NH_2-terminal hydrophilic domain, an intervening transmembrane segment and a carboxylic terminal domain having high sequence similarity with the catalytic domain of adenylate cyclases from other eukaryotes [39]. These genes are designated as rac-A and rac-B. One of these genes is expressed in *Xenopus* oocytes and have been shown to function as an adenylate cyclase. Another interesting observation was that of rac-A and rac-B mRNAs expression which was only found in promastigotes by Northern blot technique but was not detectable in amastigotes, which proves that these are developmentally regulated mRNAs [39]. So, these proteins might be involved in developmental transitions where they can function in the switch between non-infectious procyclic and infectious metacyclic promastigotes [40], or they can also interact with ligands present in the host macrophages and initiate a signal cascade leading to the differentiation of promastigotes to amastigotes.

In *T. brucei*, genes showing homology with yeast adenylate cyclase were identified and they were termed expression-site associated genes (ESAGs). Many more copies of these putative adenylate cyclases were identified and were named GRESAG4.1 and GRESAG4.2 [41]. Related adenylate cyclase genes which have actually been proved to code for functional adenylate cyclase enzymes were also identified in *T. congoense*, *T. mega*, *T. brucei gambiense*, *T. vivax* and *T. equiperdum* [42, 43]. Similar families of multigene having high homology with ESAG4 and GRESAG4.1 have been identified in *T. cruzi* and *L. donovani* are said to be sharing the common protein architecture [39]. In kinetoplastids, the cellular localization of adenylate cyclases is consistent where they act as receptors as proved by binding of antibodies against ESAG4, specifically to the cell surface along the flagella in trypanosomes [43].

The existence of receptor adenylate cyclase has also been discovered in *L. donovani* and a membrane bound RAC-A is found to be functional during exposure to phagolysosome condition (PC) which actively catalyze cAMP generation [39]. Expression of receptor adenylate cyclase mRNAs (RAC-A and RAC-B) was also found to be developmentally regulated in *Leishmania* as their expression was only found in promastigotes but not in amastigotes [39]. It has been reported that promastigotes exposed to PC shown elevated level of cAMP after 60 minutes of PC exposure which was decreased when treated with DDA (di-deoxy adenosine), an adenylate cyclase inhibitor [44]. Expression of both LdRAC-A and LdRAC-B were analyzed by immunoblot technique using anti-RAC-A and anti-RAC-B antibodies raised against *Leishmania* and their expressions were revealed in both plasma membrane and flagella. Interestingly, the expression of LdRAC-A increased significantly in PC-exposed cells after 60 minutes of exposure despite the unchanged expression profile of LdRAC-B under such condition. This result suggests that in spite of the presence of two developmentally regulated isoforms of adenylate cyclases in *Leishmania*, LdRAC-A is only functionally active during stress condition. Inducible anti-sense knock-down strategy was adopted to downregulate RAC-A and RAC-B in *L. tarentolae*, which had been successfully implemented for a number of genes earlier. Ldrac-A knocked-down parasites generated in Lt.T7TR strain of *L. tarentolae* showed no change or no elevation in intracellular cAMP level after exposure to PC. Moreover, there was a 10.2% decrease in cAMP level when RAC-A knocked-down PC-exposed parasites were further treated with DDA. Ldrac-B knocked-down parasites behaved as control parasites showing elevated cAMP levels on PC exposure. However, there was a significant decrease of cAMP level when RAC-B knocked-down cells were treated with DDA. The results indicate that LdRAC-A plays a conspicuous role in triggering cAMP response in the parasites during stress condition.

2.2 Enzymes regulating receptor adenylate cyclase function in *Leishmania*: pyrophosphatase

Pyrophosphates (PPi) are produced as by-product during the conversion of ATP to cAMP by receptor adenylate cyclase, the product accumulation of which inhibits adenylate cyclase reaction toward the formation of cAMP. PPi is found to be stored in a specialized compartment like acidocalcisomes in kinetoplastid parasites [45]. The concentration of PPi is equivalent to that of ATP in the cell in spite of its huge confinement in the acidocalcisomes of *Leishmania*. There were speculations that this high concentration of PPi (in millimolar range) might be responsible for the inhibition of cAMP production in the parasites by modulation adenylate cyclase reaction in subcellular micro domains [46]. There are at least three different pyrophosphatases present in *L. major* as revealed by genome sequence analysis. These are: membrane associated H^+-translocating pyrophosphatase (V-H^+PPase), soluble acidocalcisomal pyrophosphatase (VSP1) and an inorganic pyrophosphatase (IoPPase) and are responsible for maintaining the cAMP levels in the parasite.

Further studies have been conducted to elaborately decipher the role that RAC plays along with various molecules associated with it. PPi formed as by-product of cAMP biosynthesis inhibits adenylate cyclase function and this inhibition is reversed when PPi is hydrolysed by acidocalcisomal LdV-H^+PPase which is translocated to plasma membrane on exposure to phagolysosome condition (**Figure 2**).

Apart from the direct role of LdRAC-A in the production of cAMP during stress condition, intracellular PPi and pyrophosphatases also play a major role in regulation of cAMP concentration in the cell. *L. donovani* promastigotes were treated with foscarnet, a pyrophosphate analogue that acts as an adenylate cyclase inhibitor [47] under PC-exposed condition. PC induced cAMP generation was inhibited by foscarnet treatment after 60 minutes of PC exposure [44]. Furthermore, in PC exposed cells, total pyrophosphate pool was markedly reduced. Presence of three pyrophosphatases have been detected in *L. donovani*, namely, soluble acidocalcisomal pyrophosphatases (LdVSP1), vacuolar proton transporting pyrophosphatase (LdV-H^+PPase) and inorganic pyrophosphatase (LdIoPPase) which collectively maintain the intracellular pyrophosphate pool. Co-localization analysis with cells expressing GFP-fusion proteins of the three pyrophosphatases and acidocalcisome-targeted dye DND-lysotracker, showed little localization of LdIoPPase which was localized in cytoplasm but significant co-localization was observed for LdVSP1 and LdV-H^+PPase they were predominantly localized in the acidocalcisomes.

As revealed by immune-electron microscopic analysis, the acidocalcisomes localize in the vicinity of the cell membrane on PC exposure. PC exposure resulted in gradual decrease in intraluminal pH because of enhanced proton import by LdV-H^+PPase indicating translocation of acidocalcisome that actively imports proton, in the cell periphery following PC exposure (**Figure 2**). The translocation of acidocalcisome to membrane vicinity was further explored to find the mechanism behind such stress driven translocation. Studies clearly indicated that the movement of acidocalcisomes during stress is a microtubule and microfilament-dependent process. Pre-treatment with F-actin inhibitor, cytochalasin D, and stress exposure showed absence of acidocalcisomal translocation toward membrane. Nocodazole pre-treatment, an inhibitor of microtubule, and subsequent stress exposure also resulted in inhibition of acidocalcisomal translocation [44].

Moreover, presence of putative actin/tubulin binding proteins in *Leishmania* might provide significant clues and insight on interlinking of cytoskeletal rearrangement. One such protein has been cloned from *L. donovani* (cyclase-associated protein, LdCAP1) (Bhattacharya et al. personal communication). Unravelling the function of the same might throw light on cytoskeletal protein rearrangement

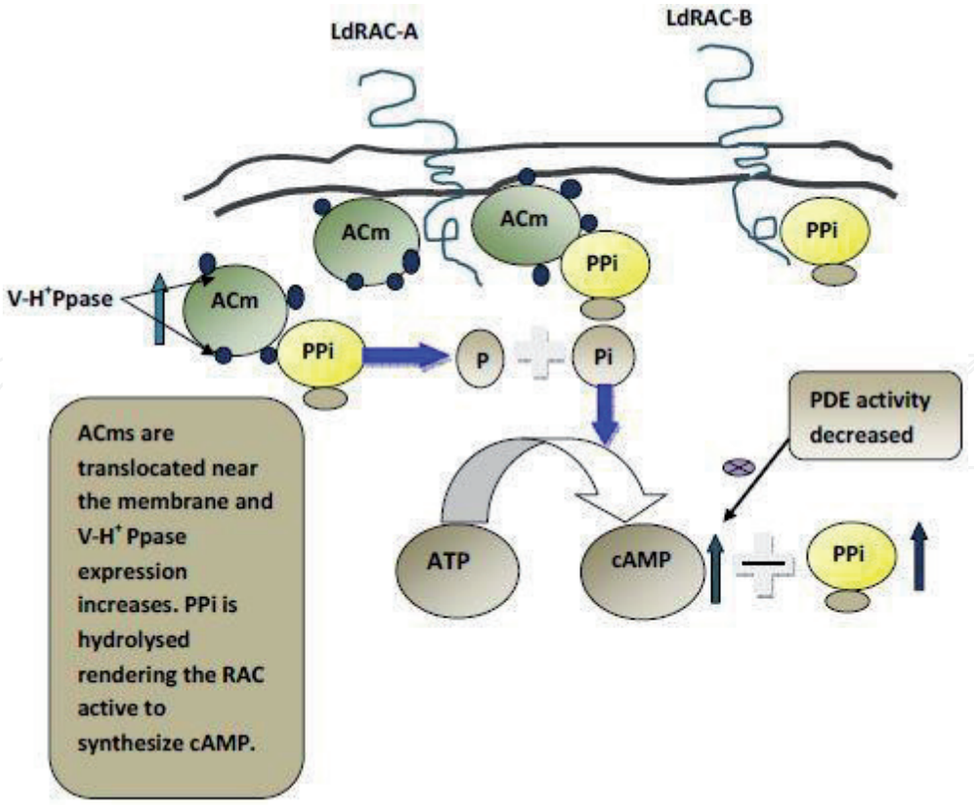

Figure 2.
Role of receptor adenylate cyclase when parasites are exposed to phagolysosomal conditions. Generally PPi inhibits adenylate cyclase function in normal conditions, but when cells are exposed to stress, the PPi are hydrolysed by acidocalcisomal V-H$^+$PPase and translocated toward membrane vicinity allowing receptor adenylate cyclase to synthesize more cAMP. In addition to this, PDE level decreases in stress-induced cells which also elevates cAMP level.

and acidocalcisomal translocation. With the translocation of acidocalcisomes in membrane vicinity, a possible co-proximal localization of membrane bound acidocalcisome (LdV-H$^+$PPase) and LdRAC-A was studied during PC exposure. No such co-localization was detected with LdRAC-B. Episomal over-expression and conditional silencing demonstrated regulatory role of V-H$^+$PPase on cAMP production. Though the direct decrease in the level of PPi by V-H$^+$PPase could not be established by the study of Biswas et al. [44], the use of PPi analogue foscarnet and the decrease in the PPi level during PC exposure indicate toward the regulation of PPi pool by this pyrophosphatase isoform. LdRAC-A, PPi pool and LdV-H$^+$PPase control intracellular cAMP level in the parasite during PC exposure.

2.3 Phosphodiesterases and intracellular cAMP signaling in *Leishmania*

Apart from pyrophosphatases that regulate the formation of intracellular cAMP by receptor adenylate cyclases, it is also important to study another dimension of cAMP regulation. Phosphodiesterases (PDEs), ubiquitous enzymes responsible for the termination of cyclic nucleotide signaling pathway by hydrolyzing cAMP to 5′-AMP or cGMP to 5′-GMP, the sole means by which the cell gets rid from the cAMP produced for controlling different cellular processes [48]. PDEs can be divided into three categories based on their catalytic properties namely, class I, class II and class III and 21 genes have been found in mammals for PDE and several in *Drosophila* and *Dictyostellium*. Though various isoforms of class I PDE have been identified in *T. brucei* and *T. cruzi*, only two PDEs have been cloned from *L. major* [48]. In *L. major* five different isoforms of PDE have been identified. Isoforms PDEB1 and PDEB2 are highly specific for cAMP and only poorly inhibited by

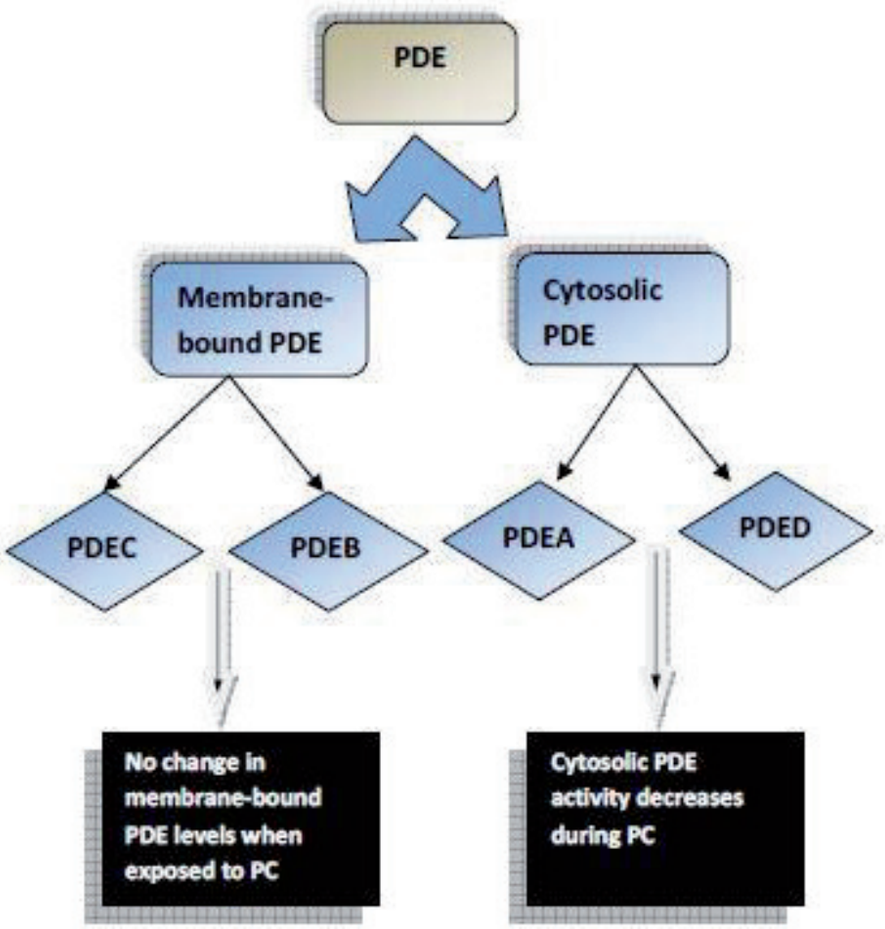

Figure 3.
PDE isoforms in Leishmania*. PDE in* Leishmania *can be categorized into membrane-bound and cytosolic PDE. On exposure to phagolysosome condition, there is no change in the expression of membrane-bound PDEs (PDEC and PDEB); but there is a significant decrease in the expression of cytosolic PDEs (PDEA and PDED).*

most inhibitors of human PDEs [48]. Crystal structure of LmjPDEB1 showed that catalytic domain of LmjPDEB1 complexed with a general PDE inhibitor, 3-isobutyl-1-methyl-xanthine (IBMX) show significant differences in binding of this inhibitor when compared to human PDEs.

Identification of different isoforms of phosphodiesterases in *L. major* indicated PDEB and PDEC to be membrane bound and PDEA and PDED to be predominantly cytosolic (**Figure 3**). LdPDEA and LdPDED were also cloned in *L. donovani* [49]. From the observation of the studies of Bhattacharya et al. [49], it has been found that the activity of cytosolic PDEs decrease during stage differentiation but the activity of membrane bound PDEs remained unchanged. From this observation, it can be inferred that PDEs might play an essential role as a controlling factor during stage differentiation of the parasites.

When cAMP-PDE activity was studied, it was found that the activity of cytosolic fraction was diminished gradually as the parasite started to differentiate into axenic amastigote stage from log phase promastigote. Protein level expression of different forms of PDEs in different stages of life cycle of *L. donovani* revealed depletion of PDEA expression in late stationary-phase promastigotes and axenic amastigotes as compared to log phase promastigotes but the expression of other PDEs such as PDEC and PDEB remain unaltered. Gradual decrease in PDEA level and its differential expression in the course of the differentiation of the parasites from promastigotes and amastigotes was observed by several experimental techniques.

2.3.1 Effect of PDEA on peroxide resistance and TSH pool

In *Leishmania*, anti-oxidant machinery plays a vital role in regulating the sustenance of the parasites in mammalian macrophages where they are exposed to oxidative stress. cAMP level elevation is linked with such phenomenon. In order to find out the functional significance of LdPDEA in such defense mechanism, LdPDEA gene was silenced using tetracycline-inducible knock-down system [49]. When PDE inhibitors were used, the parasites exhibited enhanced viability against peroxides and peroxynitrite. When cells were treated with PDE inhibitors like etazolate and trequinsin, higher resistance against peroxide and peroxynitirite was observed as compared to untreated promastigotes. Since these inhibitors are not specific for PDEA, the result of the treatment might be due to inhibition of some other forms of PDEs in the promastigotes. To ascertain the exact role of PDEA, a knock down construct was prepared to build up a tetracycline-inducible PDEA knock down system. PDEA expression was strongly reduced in both RNA and protein level after tetracycline induction and they also showed enhanced resistance against peroxide and peroxynitrite.

Peroxide neutralization is one of the major strategies of leishmanial parasite, which makes their survival possible inside the mammalian macrophage and it is done by anti-oxidant machinery of the parasite which lacks catalase. In *Leishmania*, peroxide neutralization is mainly based on trypanothione (TSH), a glutathione-spermidine conjugate, as they lack glutathione (GSH). TSH is biosynthesized from

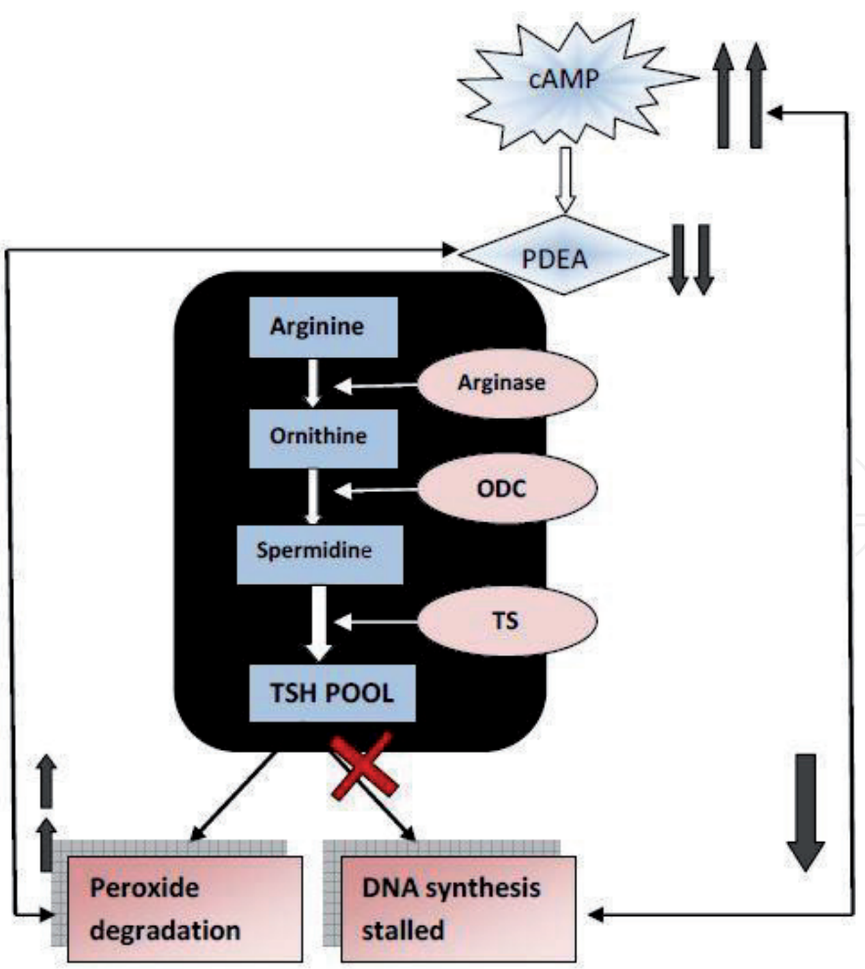

Figure 4.
Role of PDEA to control local cAMP gradient and shifting of TSH pool to peroxide neutralization.

arginine by arginase, ornithine decarboxylase and other enzymes, which converts it into spermidine and is then conjugated with GSH. No significant change in arginine and ornithine transporter was detected in PDE inhibitor treated cells and also in PDEA knocked down cells. On the contrary, when the expression of arginase and ornithine decarboxylase, the enzymes responsible for TSH biosynthesis was checked in control and PDEA inhibitor-treated cells, an increase in the expression of these enzymes was observed indicating that PDEA inhibition might have a role in TSH biosynthesis. When total thiol or intracellular TSH content was analyzed, not much alteration was observed. TSH pool is generally utilized by the parasite either for DNA replication by ribonucleotide reductase or for peroxide degradation by peroxidoxin, ascorbate peroxidase and superoxide dismutase. The expressions of enzymes responsible for peroxide degradation like peroxidoxin, superoxide dismutase and ascorbate peroxidase were elevated in PDEA-inhibited cells (**Figure 4**). Cells overexpressing PDEA also showed reduced resistance to pro-oxidants when exposed to phagolysosome condition as compared to normal cells [49].

2.3.2 Role of PDED in cAMP homeostasis

Apart from the membrane bound phosphodiesterases, a soluble, cytosolic phosphodiesterase (PDED) was cloned and characterized from *L. donovani*. Bioinformatic studies showed the presence of two pseudo-substrate sites and a putative PKA phosphorylation site at the C-terminus of PDED and PKA-mediated phosphorylation is important for the regulation of phosphodiesterase activity (**Figure 5**) [50]. It was observed that catalytic subunits of PKA (PKAC1 and PKAC2) interacts with the pseudo substrate sites of PDED after 3 hours of PC exposure. Moreover, inhibition of phosphodiesterase activity through PKA-mediated phosphorylation was observed at a further later time point of PC exposure [51]. The cytosolic localization of LdPDED was established by immunolocalization analysis using anti-LdPDED antibody which revealed its localization to be predominantly cytosolic. Interaction of LdPDED with the catalytic subunits of LdPKA within

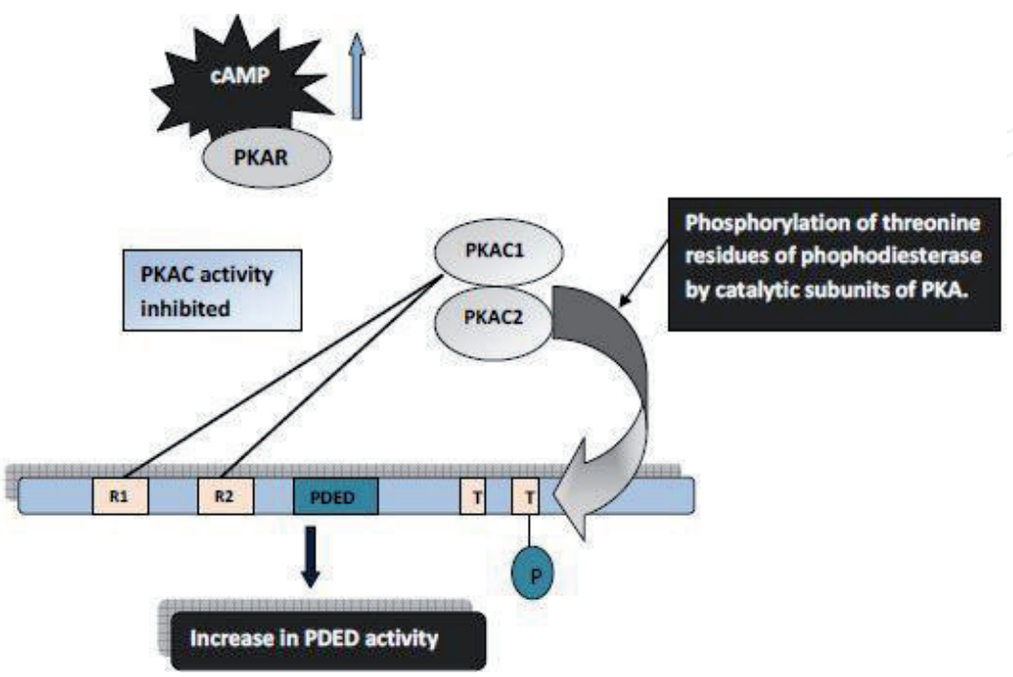

Figure 5.
LdPDED interacting with PKAC1 and PKAC2 resulting in the inhibition of kinase activity of PKA. PKA on the other hand phosphorylates threonine residue of PDED increasing its phosphodiesterase activity.

3 hours of exposure to differentiation condition leads to the inhibition of LdPKA (short-term regulation). LdPKA-mediated phosphorylation of LdPDED is observed when parasites are exposed to differentiation condition for more than 6 hours. Hydrolytic property of LdPDED is enhanced due to this phosphorylation event and this enhancement in hydrolytic activity might play a pivotal role in the maintenance of cAMP homeostasis (long term regulation) when the total cytosolic PDE activity falls because of PDEA depletion during stress condition [49]. This role of PDED in maintaining the PKA activity which in turn regulates cAMP homeostasis in the parasite during initial exposure to stress condition, might be important in the life cycle of the parasite particularly in the infection establishment within the host.

2.4 PKA as the downstream effector of cAMP in *Leishmania*

Though the existence and functioning of cAMP-dependent protein kinase (PKA) is well pronounced in eukaryotes, very little is known about the functioning of PKA in cAMP signaling of this particular parasite. PKA acts as the immediate downstream effector of cAMP in the adenylate cyclase pathway, catalyzing the transfer of γ-P from ATP to specific serine/threonine residues on the substrate protein [52]. Studies on *S. cerevisiae* reveal that one of the three PKA catalytic subunits mediates stress-induced differentiation [53]. Researches in *Dictyostelium* have suggested that cAMP is not required for differentiation if sufficient levels of PKA activity are present [54, 55] indicating profound role of PKA in differentiation. Activation of PKA by a short-term cAMP pulse induces bradyzoite differentiation, whereas a prolonged cAMP pulse inhibits differentiation [56]. It is likely that there are distinct PKA signaling pathways in the tachyzoite with opposing effects on parasite differentiation. Inhibition of PKA signals by treatment with PKA catalytic subunit inhibitor H89 induces bradyzoite differentiation [57], suggesting that PKA catalytic subunit activity may be involved in cAMP-mediated tachyzoite maintenance.

When *Leishmania* parasites were exposed to stress condition, PKA activity was significantly enhanced along with increased level of cAMP. Protein kinase activity of five different species of *Leishmania* was found to be quite high in both logarithmic and stationary phase promastigotes, being most active in *L. amazonensis* and least in *L. donovani* [58]. PKA catalytic subunits in the *Toxoplasma* genome were identified. PKA is the most important downstream effectors of cAMP signaling pathway and it exists as an holoenzyme in inactive state with the association of regulatory subunit [59–61]. In case of cAMP analog-treated cells and PC-exposed cells, substrate level phosphorylation on serine and threonine residues were also found to be increased. In most of the eukaryotic cells, PKA exist as an inactive tetrameric holoenzyme consisting of two catalytic and two regulatory subunits denoted as PKA-C and PKA-R respectively. The PKA-R subunit actually binds with cAMP causing a conformational change in the molecule resulting in the dissociation of the R and C subunits of the holoenzyme. This dissociation activates the catalytic C subunit of PKA which phosphorylates specific serine or threonine residues on substrate proteins in the cytoplasm and nucleus [62].

A 34 KD protein with similar properties of mammalian PKA-C was purified from *L. donovani* [63]. The effect of different activators and inhibitors on PKA activity was measured using promastigote lysates and fluorescent kemptide and it was found that though cAMP analogue treatment did not have any conspicuous effect on kemptide phosphorylation, treatment with PKA inhibitors like PKI and H89 profoundly decreased kemptide phosphorylation. On the other hand, PDE-resistant PKA activators increased kemptide phosphorylation when compared to basal activity. Addition of PDE inhibitors like dipyridamole and rolipram also

increased kemptide phosphorylation [64]. These results suggest that cAMP has some direct role in the activation of PKA during transformation in *Leishmania*. Treatment of promastigotes with PKA activators also resulted in growth arrest in the parasite [64]. Parasite survival in the peritoneal macrophages of Balb/c mice was examined using PKA-inhibitor treated parasites and there was a significant reduction in macrophage infection [64].

In spite of the discovery of the role played by adenylate cyclases and phosphodiesterases in cAMP homeostasis of *Leishmania*, existence of no specific cAMP-binding effector molecule was known. Bhattacharya et al. [65], in their studies, have identified a regulatory subunit of cAMP-dependent protein kinase (Ldpkar1) in *L. donovani* which was found to be homologous to class I cAMP-dependent protein kinase regulatory subunit of mammals. Studies proved beyond doubt that this regulatory subunit interact with both the catalytic subunits of PKA, thus inhibiting PKA activity. When co-immunoprecipitation assay was performed for both normal and Sp-8-Br-cAMP-pretreated cells, much weaker signal was detected for treated cells as compared to normal cells suggesting Sp-8-Br-cAMP-mediated activation of PKA. Moreover, when activity was analyzed in LdPKAR1-LdPKAC1 and LdPKAR1-LdPKAC2 immunoprecipitated complexes in the presence or absence

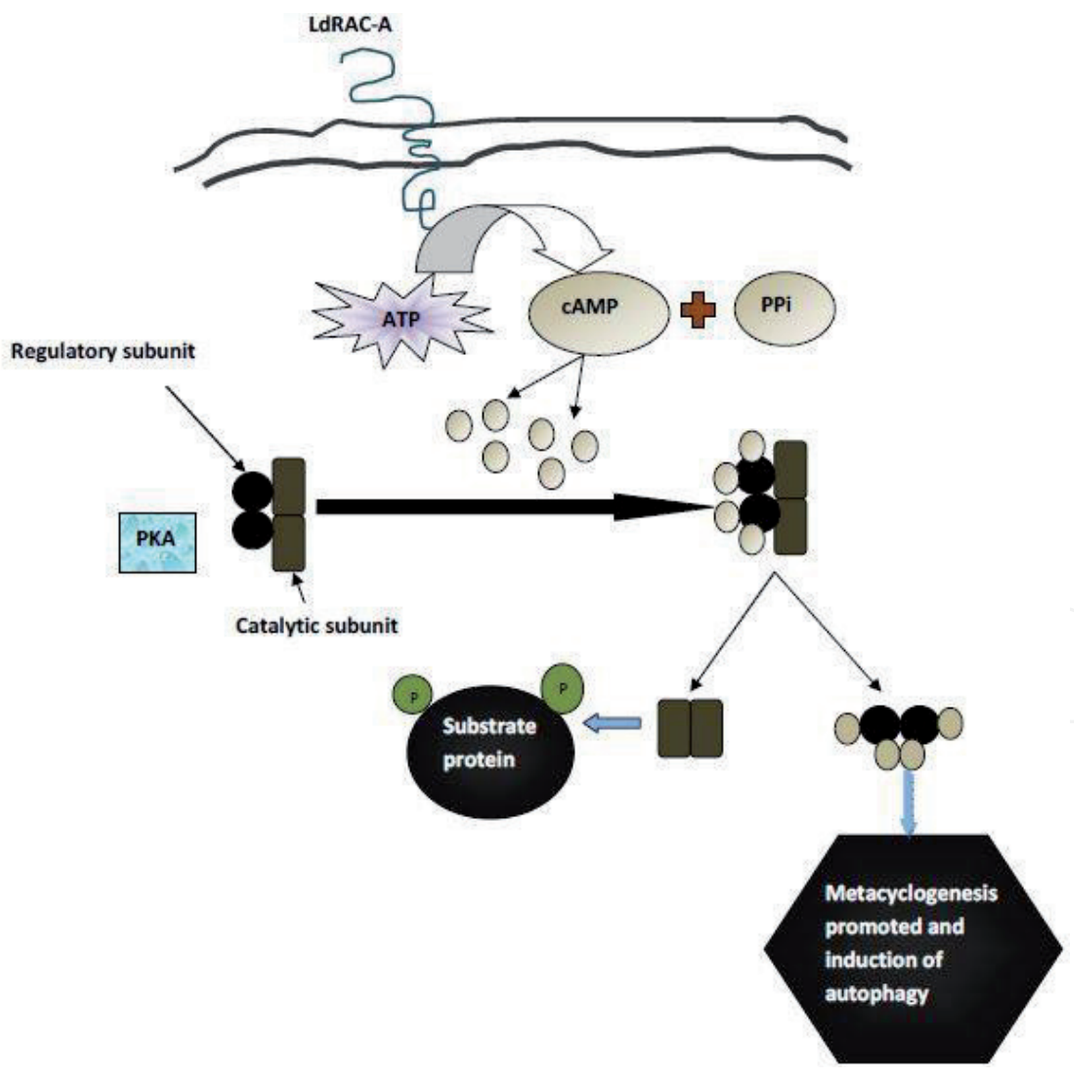

Figure 6.
cAMP-dependent PKA activity in Leishmania*: cAMP level increases on phagolysosome condition exposure and cAMP binds with the regulatory subunit of PKA enabling its dissociation from the catalytic subunit rendering the catalytic subunit active. cAMP associated regulatory subunit promotes metacyclogenesis and induces autophagy whereas activated catalytic subunit phosphorylates other proteins downstream to this signaling cascade.*

of excess cAMP, kemptide phosphorylation was increased significantly in the presence of cAMP indicating the pivotal role of cAMP in the dissociation of regulatory subunit from the catalytic subunit rendering the latter active. Moreover, the studies of Bhattacharya et al. [65] also establishes the functional importance of PKAR other than working as a cAMP effector molecule. LdPKAR1 expression was also found to be increased in late stationary phase promastigotes kept in nutrient deprived/starvation condition and metacyclogenesis, which is a pre-requisite for successful macrophage infection, was significantly induced in starved cells as compared to normal cells. Cells overexpressing LdPKAR1 also showed increased metacyclogenesis, enhanced intra-macrophage survival suggesting that LdPKAR1 overexpressed cells had greater infectivity. It can be inferred that LdPKAR1 overexpression leads to acceleration in the process of metacyclogenesis in *L. donovani* (**Figure 6**).

PKA activity assay in the presence and absence of cAMP and cGMP analogs and PKA inhibitors in both soluble fraction (SF) and membrane fraction (MF) of infective promastigotes of *L. amazonensis* showed increase in phosphorylative activity of the kinase in cAMP-analog-treated cells, and not in cGMP-analog-treated cells, was conspicuous, particularly in the SF of the promastigotes. On the contrary, PKA activity of both SF and MF of axenic amastigotes was found to be much lower as compared to that of both SF and MF of infective promastigotes under same experimental conditions [66].

Autophagy is one of the survival strategies of *Leishmania* in mammalian macrophages. Since LdPKAR1 has a direct role in the process of metacyclogenesis, its relation to autophagy was studied in the parasite. ATG8 is a marker for autophagosome formation and ATG8 tracking was done in both starved cells and in normal cells by western blot technique using polyclonal anti-LmATG8 antibody. Cells under starvation condition showed much higher level of ATG8-PE, a cleaved form of ATG8, indicating the formation of autophagosome in starved condition. When a conditional knock-down system of LdPKAR1 was constructed in *L. tarentolae*, both mRNA and protein level expression of LdPKAR1 was found to be diminished after tetracycline induction. Uninduced cells showed higher percentage of ATG8-positive structures as compared to tetracycline-induced cells. This suggested the role of PKAR1 in autophagosome formation. LdPKAR not only acts as a cAMP binding molecule in the parasite, but induce metacyclogenesis and autophagy. Studies are further required to confirm whether the process is an autophagy-induced metacyclogenesis or a metacyclogenesis-induced autophagy.

3. Conclusion

To conclude we can say that the leading researches in the recent past has enriched our knowledge on the importance of cAMP signaling in kinetoplastid parasites like *Leishmania* and their association with parasite infectivity. These findings provide insight on the functioning of different enzymes associated with cAMP metabolism (**Figure 7**). These studies point toward the fact that modulation of cAMP level in the parasite might be one of the mechanisms to control leishmaniasis and the molecules associated with the same might be tested as potent drug targets against the disease.

Presently, PDE inhibitors are potent drug targets against various human diseases. Study of human PDEs in cAMP signaling pathway has revealed their druggability in various human pathologies leading to various marketed drugs [67]. Moreover, there is a similarity between human and protozoan enzymes and in addition, the availability of human PDE inhibitors as therapeutics has thrown some light on the discovery of some specific protozoan PDE inhibitors as potential drug targets [68]. In kinetoplastid parasites like *Trypanosoma*, PDE inhibitors are being screened as potential drug targets

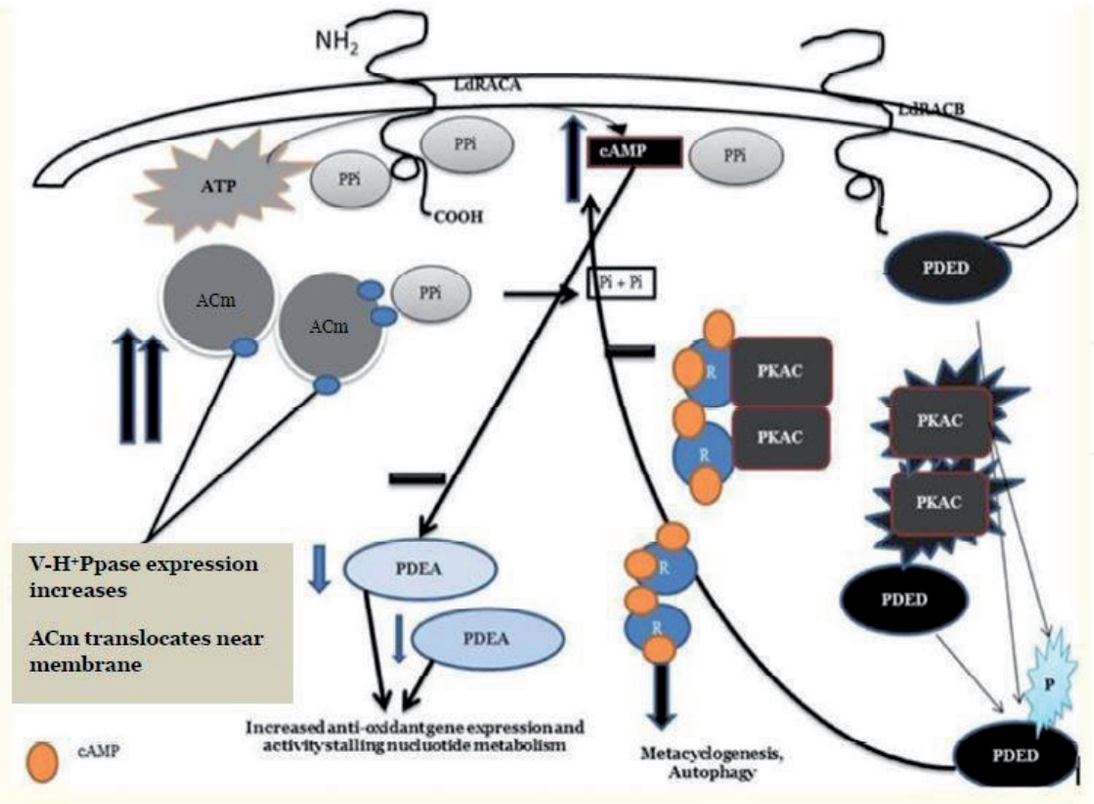

Figure 7.
An overview of cAMP signaling in Leishmania *during stress condition where receptor adenylate cyclase, PDEA, PDED, PKA the effector molecule of cAMP and acidocalcisomal pyrophosphatases play the major roles in the maintenance of cAMP homeostasis.*

and pharmacological validation of using PDEs as novel drug targets for diseases caused by the kinetoplastid parasites. This study for the first time explores the possibility of using human PDE inhibitors as the starting framework for the design of *Leishmania*-selective inhibitors. This proposal is supported by the inhibitory effects of some human PDE inhibitors observed on *T. cruzi* PDEC, e.g., etazolate inhibits human PDE4 and TcrPDEC with the IC_{50} values of 2 and 0.7 nM [69]. Among the PDE inhibitors used in this study etazolate along with rolipram showed maximum anti-proliferative activity against *Leishmania* parasite with least cytotoxic effect on macrophage cells cultured *in vitro*. Further it was observed that both of them significantly affected the G1 cell cycle arrest and mitochondrial membrane potential of the parasite, therefore we assessed *in vitro* for their ability to clear parasite load within the macrophage cells. Etazolate was found to be more effective in clearing the parasite load when the macrophage cells were pretreated with it compared to rolipram. Etazolate belongs to pyrozopolidine class compound which shows PDE4 enzyme inhibitory activity [70]. Preclinical studies as well as pharmacokinetic and safety profiles in Phase I and Phase IIa of clinical studies revealed that etazolate is a well-established drug of choice with no major side effects reported [70]. Etazolate produced antidepressant like effects in animal models of depression and at the same time it could be used in the treatment of Alzheimer's disease [71]. If through experimentation a minimal dose of etazolate could be determined then etazolate could itself be used as an anti-leishmanial drug. However, if it is not possible to determine the dose concentration which would specifically inhibit parasite PDE, then one can make use of the significant advances made in medicinal chemistry to design compounds which could specifically inhibit parasite PDE but not that of the host. This compound if developed, could act as a potential anti-leishmanial agent in future.

On the other hand, several known PDE inhibitors were tested against *Plasmodium* PDEα, and zaprinast, a known selective inhibitor of human PDE5 which is specific

for both cAMP and cGMP, turned out to be the most potent, with an IC_{50} value of 3.8 μM [72]. High concentration of PDE inhibitor like dipyridamole resulted in the inhibition of promastigote proliferation and macrophage infection in *L. major* [64].

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Conflict of interest

The authors declare no conflict of interest.

Acronyms and abbreviations

AC	acidocalcisomes
cAMP	cyclic adenosine monophosphate
PDE	phosphodiesterase
PKA	protein kinase A
PPi	inorganic pyrophosphate
RAC	receptor adenylate cyclase

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