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1α,25(OH)$_2$D$_3$ and parathyroid hormone (PTH) signaling in rat intestinal cells: activation of cytosolic PLA2☆

Claudia Gentili, Susana Morelli, Ana Russo de Boland*

Department of Biologia, Bioquimica and Farmacia, Universidad Nacional del Sur, San Juan 670, Bahia Blanca 8000, Argentina

Abstract

In the current study, we have probed the role of cytosolic phospholipase A2 (cPLA2) activity in the cellular response to the calciotropic hormones, 1α,25,dihydroxy-vitamin D$_3$ [1α,25(OH)$_2$D$_3$] and PTH. Stimulation of rat enterocytes with either hormone, increased release of arachidonic acid (AA) [^{3}H-AA] one–two fold in a concentration and time-dependent manner. The effect of either hormone on enterocytes was totally reduced by preincubation with the intracellular Ca^{2+} chelator BAPTA-AM (5 μM), suggesting that the release of AA following cell exposure to the calciotropic hormones occurs mainly through a Ca^{2+}-dependent mechanism involving activation of Ca^{2+}-dependent cPLA2. Calciotropic homone stimulation of rat intestinal cells increases cPLA2 phosphorylation (three to four fold). This effect was decreased by PD 98059 (20 μM), a MAP kinase inhibitor, indicating that this action is, in part, mediated through activation of the MAP kinases ERK 1 and ERK2. Enterocytes exposure to 1α,25(OH)$_2$D$_3$ (1 nM) or PTH (10 nM) also resulted in P-cPLA2 translocation from cytosol to nuclei and membrane fractions, where phospholipase subtrates reside. Collectively, these data suggest that PTH and 1α,25(OH)$_2$D$_3$ activate in duodenal cells, a Ca^{2+}-dependent cytosolic PLA2 and attendant arachidonic acid release and that this activation requieres prior stimulation of intracellular ERK1/2. 1α,25(OH)$_2$D$_3$ and PTH modulation of cPLA2 activity may change membrane fluidity and permeability and thereby affecting intestinal cell membrane function.

Keywords: 1α,25,Dihydroxy-vitamin D$_3$; PTH; Rat enterocytes; cPLA2; Arachidonic acid; Signal transduction

1. Introduction

The hormonal form of Vitamin D$_3$, 1α,25(OH)$_2$D$_3$, plays a major role in regulating calcium homeostasis together with PTH and calcitonin. As in other target cells [1], 1α,25(OH)$_2$D$_3$ elicits its actions in intestine through nuclear Vitamin D receptor (VDR) mediated gene trascription and a non-genomic transmembrane signal transduction mechanism. The transduction of 1α,25(OH)$_2$D$_3$ and PTH signals through the plasma membrane of rat intestinal cells (enterocytes) involve G protein-coupled stimulation of adenylyl cyclase and phospholipase C to yield the second messengers cAMP, inositol triphosphate (IP3) and diacylglycerol (DAG) and activation of protein kinases A and C, which results in the fast stimulation of Ca^{2+} mobilization from inner stores and Ca^{2+} influx through voltage-dependent Ca^{2+} channels [2–6]. In addition, both hormones stimulate in intestinal cells the tyrosine phosphorylation of phospholipase Cγ and the MAP kinases ERK1 and ERK2 [7,8].

Phospholipase A2 (PLA2) catalyzes the hydrolysis of the sn-2 position of membrane glycerophospholipids to liberate arachidonic acid (AA), a precursor of eicosanoids including prostaglandins and leukotrienes. The same reaction also produces lysophosholipids, which represent another class of lipid mediators. So far, at least 19 enzymes that possess PLA2 activity have been identified and cloned in mammals [9]. The secretory PLA2 (sPLA2) family, implicated in a number of biological processes, such as modification of eicosanoid generation, inflammation, and host defense. The cytosolic Ca^{2+}-depedent PLA2 (cPLA2) family, among which cPLA2α has been paid much attention by researchers as an essential component of the initiation of AA metabolism and the Ca^{2+}-independent PLA2 (iPLA2) family which may play a major role in phospholipid remodeling. Activation of PLA2 by 1α,25(OH)$_2$D$_3$ was demostrated in other cell types, such as skeletal muscle cells [10], chondrocytes [11] and hepatocytes [12]. Furthermore, stimulation of cPLA2 by PTH has been reported in renal cells [13].

To further elucidate the signal trasduction pathways activated by 1α,25(OH)$_2$D$_3$ and PTH in rat intestinal cells, we

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* Corresponding author. Tel.: +54-91-595-100x24; fax: +54-91-595-130.
E-mail address: aboland@criba.edu.ar (A.R.d. Boland).

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have investigated whether these hormones activate cytosolic phospholipase A2 and the mechanism leading to its activation and release of arachidonic acid.

2. Materials and methods

2.1. Materials

Synthetic rat PTH (1–34), leupeptin, aprotinin, Immobilon P (Polyvinylidene difluoride, PVDF) membranes, were from Sigma Chemical Co. (St. Louis, MO, USA). PD98059 was obtained from Calbiochem (San Diego, CA, USA). [5,6,8,9,11,12,14,15,-^{3}H] arachidonic acid (specific activity, 216 Ci/mmol) and ECL Western blot analysis system were purchased from Pharmacia Biotech for Amersham (Arlington Heights, IL, USA). All other reagents were of analytical grade.

2.2. Animals

Three-month-old male Wistar rats were fed with standard rat food (1.2% Ca; 1.0% phosphorus), given water *ad libitum* and maintained on a 12 h light 12 h dark cycle. Animals were sacrificed by cervical dislocation.

2.3. Isolation of duodenal cells

Enterocytes were isolated from 3 months old Wistar rats duodenum as previously described [4].

2.4. Assessment of arachidonic acid mobilization

Release of [^{3}H] from duodenum prelabeled with [^{3}H]AA was used to assess the response to the hormones. The duodenum was placed in the incubation medium (154 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 1 mM $MgCl_2$, 10 mM NaMOPS (pH 7,4), 5.6 mM glucose, 1 mM $CaCl_2$) with 1 μCi/ml [^{3}H] arachidonic acid for 2 h at 37 °C under O_2/CO_2 (95:5%) atmosphere. After extensive washing with phosphate-buffered saline, they were incubated at 37 °C in fresh medium plus 0.5% BSA, agonists, antagonists or vehicle. The supernatants were removed at the indicated times and centrifuged at 10.000 × *g* for 5 min to remove floating cells and/or cell debris. The radioactivity of the media was quantified by scintillation counting. The results were normalized and expressed as a percentage of the mean of the basal release.

2.5. Electrophoresis and Western blotting

Following treatment, the enterocytes were lysed in ice-cold RIPA buffer [1% Nonidet P40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM NaF, 10 mg/ml of aprotinin, 10 mg/ml of leupeptin, and 50 mM Tris–HCl (pH 7.4)]. Lysate proteins (25 μg) were resolved by one-dimensional SDS-PAGE [14] and then transferred to Immobilon P (PVDF) membranes as previously described [7]. The membranes were then probed with anti-phospho Ser 505-cPLA2 monoclonal antibody. The anti-phospho-cPLA2 antibody was then stripped and the membrane was reprobed with an antibody that recognizes total cPLA2 to account for equal loading. Autoradiograms were scanned with a Hewlett Packard densitometer to quantitate cPLA2 signals as previously described [7].

2.6. Statistical evaluation

The significance of the results was evaluated by Student's *t*-test [15].

3. Results and discussion

This study demonstrates that the calciotropic hormones, 1α,25(OH)$_2$D$_3$ and PTH, rapidly stimulate arachidonic acid release in rat duodenal cells. Shown in Fig. 1 are the time-courses of the release of [^{3}H]AA into media from rat enterocytes prelabeled with [^{3}H]AA. In the presence of 1α,25(OH)$_2$D$_3$ (10^{-9} M) or PTH (10^{-8} M), the release of [^{3}H]AA was about 48% or 52% over the control value at 2 min, and 100% or 158% at 15 min, respectively. The effect of PTH was dose-dependent, with maximal

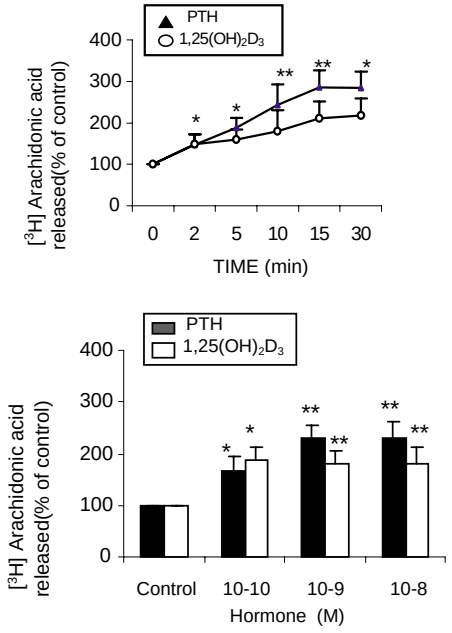

Fig. 1. Release of [^{3}H] arachidonic acid induced by 1α,25(OH)$_2$D$_3$ and PTH in rat intestinal cells. Rat duodenum was prelabelled with [^{3}H]AA as described under Methods, followed by incubation with 1α,25(OH)$_2$D$_3$ or PTH. Radioactivity released into media was measured. Data represent the average of 3 independent experiments performed in quatriplicate. Means ± S.D. are given. $*P < 0.05$, $**P < 0.025$ with respect to its corresponding control.

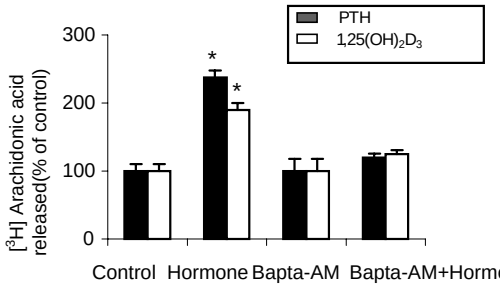

Fig. 2. [^{3}H] arachidonic acid release dependent of 1α,25(OH)$_2$D$_3$ and PTH is suppressed by the intracellular Ca^{2+} chelator BAPTA-AM. Release of [^{3}H]AA was measured with or without BAPTA-AM (5 μM) for 10 min in the presence or absence of 1α,25(OH)$_2$D$_3$ (10^{-9} M) or PTH (10^{-8} M). Data represent the average of 2 independent experiments performed in quatriplicate. Means ± S.D. are given. $*P < 0.025$ with respect to its corresponding control.

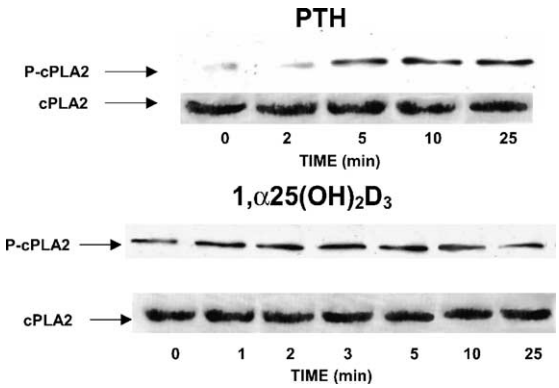

Fig. 3. cPLA2 is phosphorylated on serine 505 after stimulation of rat enterocytes with 1α,25(OH)$_2$D$_3$ or PTH. Cells were treated with 1α,25(OH)$_2$D$_3$ (10^{-9} M) or PTH (10^{-8} M) for the indicated times. Upon reaction termination, cells were sonicated and soluble fractions were subjected to SDS-PAGE. The resulting immunoblots were probed with anti-phospho serine 505 cPLA2 antibody. The immunoblots were stripped and re-probed with anti-cPLA2 antibody. Results are representative of three independent experiments.

stimulation achieved at 10^{-8} to 10^{-9} M. No major differences were detected in cells stimulated with 1α,25(OH)$_2$D$_3$ between 10^{-10} and 10^{-8} M. As shown in Fig. 2, the effect of both hormones on rat enterocytes [^{3}H]AA release is Ca^{2+}-dependent, as is potently inhibited by the intracellular Ca^{2+} chelator BAPTA-AM (5 μM). These results suggest that hormone-dependent AA release occurs mainly through a Ca^{2+}-dependent mechanism involving activation of Ca^{2+}-dependent cPLA2 and are consistent with the notion that either hormone increase intracellular Ca^{2+} in duodenal cells [16,17].

The important role of arachidonic acid in cellular activation ensures that its levels are tightly controlled. cPLA2 plays a role in maintaining arachidonate levels and is subject to complex mechanisms of regulation at both the transcriptional and post-translational levels. Agonist-induced phosphorylation of cPLA2 on serine residues has been verified, and the increased cPLA2 activity in stimulated cells is attributed to phosphorylation on Ser 505 by MAPK [18]. Phosphorylation of Ser 505 is important in the activation of cPLA2 *in vivo* since overexpression of mutant cPLA2 in Chinese hamster ovary cells fails to enhance agonist-induced AA release as seen when wild type enzyme is expressed [19]. As a first step in elucidating the mechanism underlaying the stimulation of cPLA2 activity, we evaluated whether the hormones affect the phosphorylation of cPLA2. To that end, the cells were stimulated with 1α,25(OH)$_2$D$_3$ or PTH. After reaction termination, cells were sonicated and cytosolic fractions subjected to SDS-PAGE. Western blots were performed using anti-phospho-Ser 505 cPLA2. The results summarized in Fig. 3 clearly show, that PTH, and to a less extent, 1α,25(OH)$_2$D$_3$ induced the phosphorylation of cPLA2 on serine 505. cPLA2 serine-phosphorylation was totally suppressed by chelation of intracellular calcium with 5 μM BAPTA-AM (Fig. 4A), confirming the involvement of the Ca^{2+}-dependent cytosolic PLA2. Since in enterocytes both hormones activate the MAP kinase pathway (ERK1 and ERK2) [7,8], we studied the role of ERKs in mediating 1α,25(OH)$_2$D$_3$ and PTH serine-phosphorylation

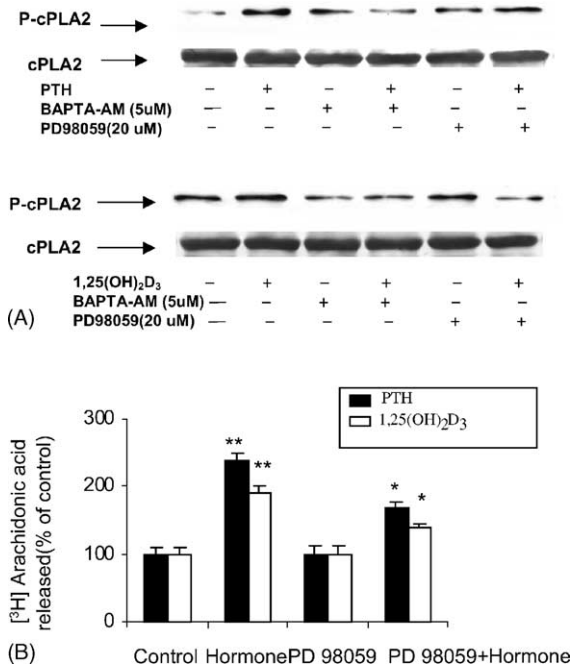

Fig. 4. Effect of BAPTA-AM and MAP kinase inhibitor PD 98059 on 1α,25(OH)$_2$D$_3$ and PTH-induced cPLA2 serine-phosphorylation and [^{3}H]AA release. Rat enterocytes were treated with BAPTA-AM (A) or PD98059 (A and B) before stimulation by 1α,25(OH)$_2$D$_3$ (10^{-9} M) or PTH (10^{-8} M) for 10 min. Lysates were immunoblotted with anti-phospho serine 505 cPLA2 antibody (A). The exposures shown are representative of three similar results. [^{3}H]AA release (B) was determined as described in legend to Fig. 1. Data represent the average of two independent experiments performed in quatriplicate. Means ± S.D. are given. $*P < 0.05$, $**P < 0.025$ with respect to its corresponding control.

of cPLA2 and [^{3}H]AA release, and analysed the effect of the specifc MEK inhibitor PD-98059. As shown in Fig. 4A and B, preincubation of enterocytes with 20 μM PD-98059 inhibited to a great extent both 1α,25(OH)$_2$D$_3$ and PTH-induced cPLA2 phosphorylation and [^{3}H]AA release. This suggests that ERKs are part of the mechanism by which 1α,25(OH)$_2$D$_3$ and PTH activate cPLA2 in rat enterocytes. Other kinases, such as PKC and protein kinase A can phosphorylate cPLA2 in vitro, but this does not result in a significant increase in cPLA2 activity. However, there is evidence for a role for PKC in the activation of PLA2 and regulation of AA release [20]. PKC activation can play a role in 1α,25(OH)$_2$D$_3$ and PTH effects by triggering a kinase cascade leading to MAPK activation [21]. The contribution of other MAPK members, such as p38 MAPK, should not be discarded and will be the subject of future investigations.

cPLA2 is subject to diverse mechanisms of regulation that we are only beginning to understand. cPLA2 requires calcium for activity; however, unlike sPLA2, calcium is necessary for binding cPLA2 to membrane or phospholipid vesicles rather than for catalysis [22]. cPLA2 contains an N-terminal calcium-dependent phospholipid binding domain that shares homology with the C2 domains in the conventional isoforms of PKC [23]. The location of phosphorylated cPLA2 in cytosol and membrane fractions in resting and in enterocytes stimulated by 1α,25(OH)$_2$D$_3$ and PTH was studied by immunoblot analysis. To that end, cells were stimulated with either hormone and the cytosol and particulate fractions were isolated by ultracentrifugation. Proteins in each fraction were separated by SDS-PAGE, transferred to immobilon membranes and immunoblotted with anti-phospho Ser 505-cPLA2 antibody. As shown in Fig. 5, phospho-cPLA2 was poorly detected in resting cells. On activation with either 1α,25(OH)$_2$D$_3$ or PTH, phospho-cPLA2 appears within 5 min in the nuclei and plasma membranes of enterocytes, where its substrates reside.

The results of this study demonstrate that the calciotropic hormones 1α,25(OH)$_2$D$_3$ and PTH stimulate the rapid release of AA from rat enterocytes and that the ERK1/2 pathway and increases in intracellular calcium act in concert to stimulate the cPLA2 enzyme, resulting in the liberation of AA. 1α,25(OH)$_2$D$_3$ and PTH modulation of cPLA2 activity may change membrane fluidity and permeability and thereby affecting intestinal cell membrane function.

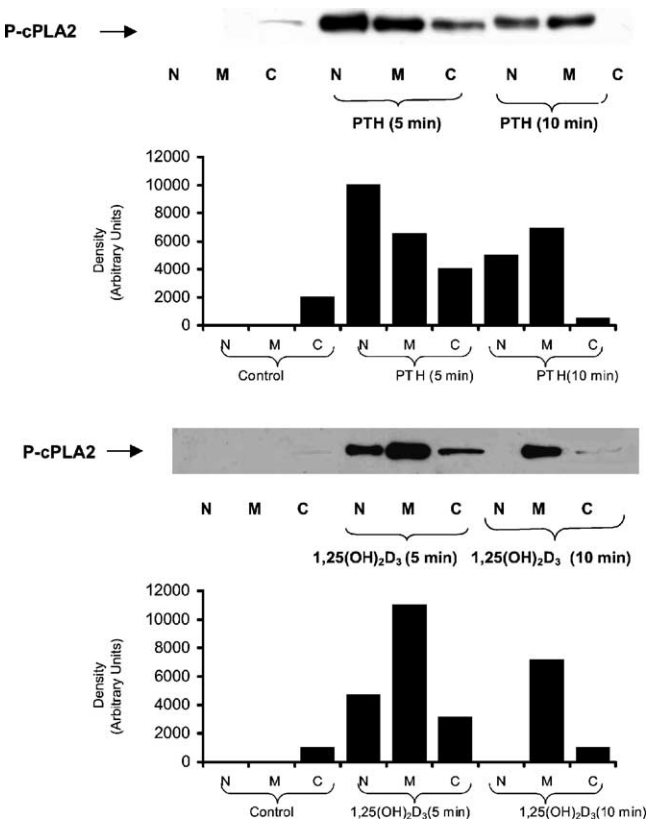

Fig. 5. cPLA2 translocates to enterocyte microsomal membranes and nuclei in response to 1α,25(OH)$_2$D$_3$ and PTH. After treatment with 1α,25(OH)$_2$D$_3$ (10^{-9} M) or PTH (10^{-8} M), cells were homogenized and separated into cytosol, nuclei and membrane fractions by centrifugation. Equal portions of each fraction were subjected to SDS-PAGE and immunoblotted with anti-phospho serine 505 cPLA2 antibody. Results are representative of three independent experiments.

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