# PTH and Phospholipase A2 in the Aging Process of Intestinal Cells

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**Abstract**    In this study we analyzed, for the first time, alterations in phospholipase A2 (PLA2) activity and response to parathyroid hormone (PTH) in rat enterocytes with aging. We found that PTH, rapidly stimulate arachidonic acid (AA) release in rat duodenal cells (+1- to 2-fold), an effect that is greatly potentiated by aging (+4-fold). We also found that hormone-induced AA release in young animals is $Ca^{2+}$-dependent via cPLA2, while AA released by PTH in cells from aged rats is due to the activation of cPLA2 and the $Ca^{2+}$-independent PLA2 (iPLA2). In enterocytes from 3 months old rats, PTH induced, in a time and dose-dependent fashion, the phosphorylation of cPLA2 on serine 505, with a maximun at 10 min (+7-fold). Basal levels of cPLA2 serine-phosphorylation were higher in old enterocytes, affecting the hormone response which was greatly diminished (+2-fold at 10 min). cPLA2 phosphorylation impairment in old animals was not related to changes of cPLA2 protein expression and did not explain the substantial increase on PTH-induced AA release with aging, further suggesting the involvement of a different PLA2 isoform. Intracellular $Ca^{2+}$ chelation (BAPTA-AM, 5 μM) suppressed the serine phosphorylation of cPLA2 in both, young and aged rats, demonstrating that intracellular $Ca^{2+}$ is required for full activation of cPLA2 in enterocytes stimulated with PTH. Hormone effect on cPLA2 was suppressed to a great extent by the MAP kinases ERK 1 and ERK2 inhibitor, PD 98059 (20 μM), the cAMP antagonist, Rp-cAMP, and the PKC inhibitor Ro31820 both, in young and aged animals. Enterocytes exposure to PTH also resulted in phospho-cPLA2 translocation from cytosol to nuclei and membrane fractions, where phospholipase subtrates reside. Hormone-induced enzyme translocation is also modified by aging where, in contrast to young animals, part of phospho-cPLA2 remained cytosolic. Collectively, these data suggest that PTH activates in duodenal cells, a $Ca^{2+}$-dependent cytosolic PLA2 and attendant AA release and that this activation requires prior stimulation of intracellular ERK1/2, PKA, and PKC. cPLA2 is the major enzyme responsible for AA release in young enterocytes while cPLA2 and the $Ca^{2+}$-independent iPLA2, potentiate PTH-induced AA release in aged cells. Impairment of PTH activation of PLA2 isoforms upon aging may result in abnormal hormone regulation of membrane fluidity and permeability and thereby affecting intestinal cell membrane function. J. Cell. Biochem. 93: 312–326, 2004.    © 2004 Wiley-Liss, Inc.

**Key words:** PTH; rat enterocytes; PLA2; arachidonic acid; aging; signal transduction

Phospholipase A2 (PLA2) superfamily includes a large group of extracellular and intracellular enzymes that catalyze the hydrolysis of the sn-2 fatty acyl bond of phospholipids to yield free fatty acids and lysophospholipids

[Dennis, 1994]. PLA2 constitutes the main metabolic route by which fatty acids such as arachidonic acid (AA) are liberated from their lipid storage sites for the synthesis of eicosanoids, including prostaglandins and leukotrienes. The other reaction products, lysophospholipids such as lysophosphatidic acid and lysophosphatidylcholine, are also biologically active by themselves and are the precursors of other potent bioactive mediators, such as platelet-activating factor [Kudo and Murakami, 2002]. In mammalian cells, as many as five different extracellular (secreted) PLA2s (sPLA2) exist: groups I, IIA, IIC, V, and X. They require millimolar calcium concentrations for catalytic activity, and do not manifest significant fatty acid selectivity in vitro [Dennis, 1994]. The intracellular PLA2s are further divided into

groups IV (cytosolic $Ca^{2+}$ dependent PLA2 or cPLA2) and VI (intracellular $Ca^{2+}$ independent PLA2 or iPLA2) based on the $Ca^{2+}$ requirements needed for basal activity [Dennis, 1994]. Until now, cPLA2 $\alpha$, $\beta$, and $\gamma$ isoforms have been cloned [Underwood et al., 1998; Pickard et al., 1999; Song et al., 1999], they have well conserved catalytic sites containing a serine residue at the catalytic center. In spite of been named cytosolic, cPLA2$\gamma$ is constitutively membrane-bound [Underwood et al., 1998]. In general, activation of cPLA2 is tightly controlled by cytoplasmic $Ca^{2+}$ levels and by phosphorylation following various stimuli [Clark et al., 1995; Leslie, 1997; Hirabayashi and Shimizu, 2000]. It requires millimolar $Ca^{2+}$ for membrane translocation, where its phospholipid substrate is located, but not for catalysis [Leslie, 1997], and possesses a preference for phospholipids containing AA [Mayer and Marshall, 1993]. iPLA2 exhibits no substrate specificity for AA-containing phospholipids and no $Ca^{2+}$ requirement for activity [Ackermann and Dennis, 1995]. Coexpression of different forms of PLA2 has been found within the same cell or tissue [Balsinde et al., 1999; Yang et al., 1999], however the physiological functions of individual PLA2s in rat intestine are currently unknown. Under normal conditions, products of PLA reactions (AA and its metabolites) are closely associated with cell growth, regeneration, and development [Smalheiser et al., 1996; Liu and Levy, 1997]. Accumulating evidence suggests that cPLA2 can translocate from the cytosolic compartment to the nuclear membrane [Glover et al., 1995; Schievella et al., 1995], and in human monocytes, cPLA2 physically associates with a transcriptional factor, which facilitates its redistribution into the nucleus to regulate c-Myc expression [Tashiro et al., 2004].

Phospholipases, particularly PLA2, are key factors in the membrane hypothesis of aging [Zs-Nagy et al., 1988]. In this regard, PLA2 plays a major role in phospholipids membrane destabilization, the synthesis of inflammatory mediators, and the generation of and/or response to free radicals [Rosenthal and Franson, 1989]. Free fatty acids released by PLA2 are converted to inflammatory mediators such as leukotrienes and prostaglandins, and in this process, free radicals are formed which in turn can damage more membrane phospholipids. In addition the lyso derivatives released by PLA2

action have detergent effects which can also damage cell membranes [Phillips et al., 1965]. There is evidence that parathyroid hormone (PTH) stimulates PLA2 activity in renal cells [Sheu et al., 1997]. Furthermore, we have recently obtained evidence that the hormone increased the release of radioactive AA in prelabeled intestinal cells via $Ca^{2+}$-dependent cPLA2 activity [Gentili et al., 2003]. Intestinal cells exposure to PTH alters $Ca^{2+}$ homeostasis, and this appears to be an important initial signal for cPLA2 activity. In these cells, PTH is known to increase intracellular free $Ca^{2+}$ concentrations through either voltage-dependent $Ca^{2+}$ channels or by release of intracellular calcium [Gentili et al., 2004]. The present study is designed to explore age-related changes in the mechanism of PLA2 activation by PTH in rat intestinal cells.

## MATERIALS AND METHODS

### Materials

Synthetic rat PTH (1–34), leupeptin, aprotinin, Immobilon P (Polyvinylidene difluoride, PVDF) membranes, were from Sigma Chemical Co. (St. Louis, MO). PD98059 was obtained from Calbiochem (San Diego, CA). [5,6,8,9,11,12,14,15,-$^3$H] AA (specific activity, 216 Ci/mmol) and ECL Western blot analysis system were purchased from Pharmacia Biotech for Amersham (Arlington Heights, IL). Anti-phospho cPLA2 and anti-cPLA2 were from Cell Signaling Technology, Inc. (Beverly, MA). All other reagents were of analytical grade.

### Animals

Male Wistar rats (3- and 24-month-old) 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.

### Isolation of Duodenal Cells

Duodenal cells were isolated as described previously [Picotto et al., 1996]. The method employed yields preparations containing only highly absorptive epithelial cells that are devoid of cells from the upper villus or crypt [Weiser, 1973]. The duodenum was excised, washed with 0.9% NaCl and trimmed of adhering tissue. The intestine was slit lengthwise and cut into small segments (2 cm length) and placed into solution A containing (in mM): 96 NaCl, 1.5 KCl,

8 $KH_2PO_4$, 5.6 $Na_2HPO_4$, 27 Na citrate, pH 7.3, for 10 min at 37°C. The solution was discarded and replaced with isolation medium containing (mM): 154 NaCl, 10 $NaH_2PO_4$, 1.5 EDTA, 0.5 dithiothreitol, 5.6 glucose, pH 7.3, for 15 min at 37°C with continuous shaking (87 oscillations/min). The cells were sedimented by centrifugation at 750 × g for 10 min, washed twice with 154 mM NaCl, 10 mM $NaH_2PO_4$, 5.6 mM glucose, pH 7.3, and then resuspended in measurement buffer (see below). All the steps mentioned above were performed under an atmosphere of 95% $O_2$:5% $CO_2$. Cell viability was assessed by the Trypan Blue technique. Exclusion of the dye in >90% of the cells was observed for at least 90 min after isolation. This method yields preparations containing highly absorptive ephitelial cells, devoid of cells from the upper villus or the crypt. Enterocytes isolated by this procedure have been shown to possess functional characteristics of intestinal cells [Weiser, 1973]. Morphological characterization of preparations was performed by phase-contrast microscopy and revealed no morphological differences between enterocytes isolated from young and old rats.

## Cellular Fractionation

Scrapped duodenal mucosae of young and aged rats were collected in buffer A (20 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 1 mM EDTA, 20 mM NaF, 0.5 nM phenylmethylsulfonyl fluoride (PMSF), 0.2 mM $Na_2VO_3$, 2 μg/ml leupeptin, and 2 μg/ml aprotinin), homogenised in a teflon-glass hand homogenizer (20 stokes) and centrifuged at 1000 × g for 15 min, 4°C. The pellet (nuclei) was washed twice in buffer A and resuspended in 400 μl of buffer lysis (50 mM Tris-HCl pH, 7.4, 150 mM NaCl, 2 mM EGTA, 1% nonidet P40, 0,25% Na deoxycholate with protease inhibitors: 1 mM PMSF, 20 μg/ ml leupeptin and 20 μg/ml aprotinin and phosphatase inhibitors: 0.2 mM $Na_3VO_3$, 25 mM NaF). The supernatant was centrifuged at 12,000 × g for 15 min, 4°C and the resulting pellet (mitochondrial fraction) was discarded. The supernatant was then subjected to ultracentrifugation at 100,000 × g for 1 h, 4°C for isolation of the cytosolic (supernatant) and microsomal (pellet) fractions. All fractions were sonicated to uniformity and immediately frozen and stored at −80°C until used. The protein content of the subcellular fractions was measured according to Lowry et al. [1951]. In order to ensure the purity of the isolated fractions, Lamin B, Gαq, and cytosolic cSrc were used as markers of nuclei, membrane, and cytosol, respectively.

## Assessment of AA Mobilization

Release of [³H] from duodenum prelabeled with [³H]AA was used to assess the response to PTH. 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 [³H] AA 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.

## 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 μg/ml of aprotinin, 10 μg/ml of leupeptin, and 50 mM Tris-HCl (pH 7.4)]. Lysate proteins (25 μg) were resolved by one-dimensional SDS−PAGE [Laemmli, 1970] and then transferred to Immobilon P (PVDF) membranes. The membranes were immersed in TBS buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl), containing 5% skim milk for 2 h to block nonspecific binding. Anti-phospho (ser 505) cPLA2 antibody was allowed to react with the membrane overnight at 4°C. The membranes were then twice washed (5 min) with TBS-T (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 20), followed by one 10-min wash with TBS-T. The membranes were incubated with 1 μg/ml of peroxidase labeled goat anti-rabbit IgG antibody in TBS-T for 1 h at room temperature. After two washes with TBS-T, the membrane was visualized by using an enhanced chemiluminiscent technique (ECL, Amersham Corp.), according to the manufacturer's instructions. 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. Images were obtained with a model GS-700 Imaging Densitometer from Bio-Rad (Hercules, CA) by scanning at 600 dpi and printing at the same resolution. Bands were quantified using the Molecular Analyst program (Bio-Rad).

### Statistical Evaluation

The significance of the results was evaluated by Student's $t$-test [Snedecor and Cochran, 1967].

### RESULTS

In this study, we analyzed for the first time, alterations in PLA2 activity and response to PTH in rat enterocytes with aging. We first tested the release of AA, the major product of PLA2 activity. To that end, duodena isolated from 3- and 24 month-old rats were prelabeled with [³H]AA and briefly stimulated with PTH ($10^{-8}$ M). Figure 1A shows the time-courses of the release of [³H]AA into media from pre-labeled enterocytes. Treatment with PTH increased the release of [³H]AA about 1-fold over
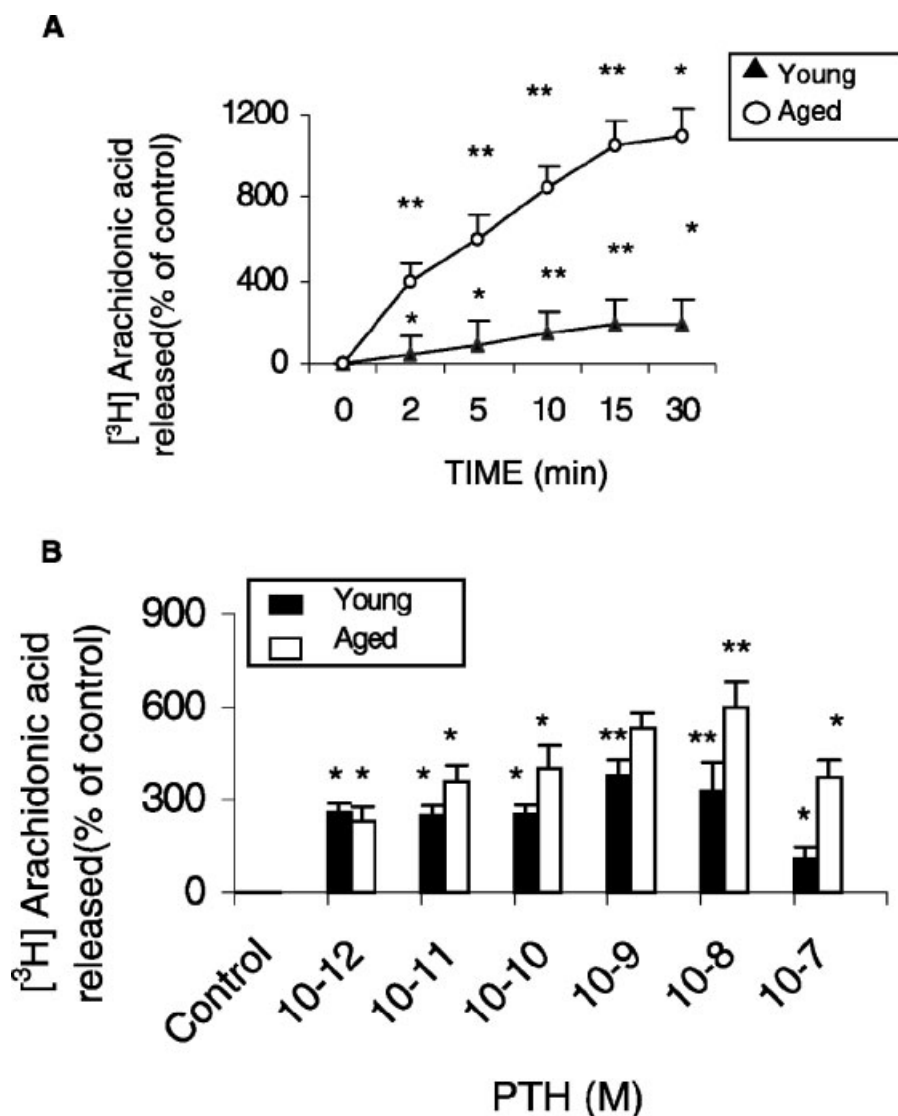


**Fig. 1.** Time and dose profile of [³H] arachidonic acid (AA) release induced by PTH in rat intestinal cells. Rat duodenum from young (3 months) and aged (24 months) was prelabeled with [³H]AA as described under "Materials and Methods," followed by incubation with PTH ($10^{-8}$ M) for the indicated times (**A**) or with PTH ($10^{-12}$–$10^{-7}$ M) for 15 min (**B**). Radioactivity released into media was measured. Data represent the average of three independent experiments performed in quadruplicate. Mean ± SD are given. *$P < 0.05$, **$P < 0.025$ with respect to its corresponding control.

the control value at 2 min, and 2-fold at 15 min. With aging hormone-dependent [3H]AA release was substantially enhanced (+4-fold at 2 min and +9-fold at 15 min). The effect of PTH was dose-dependent, with maximal stimulation achieved at $10^{-8}$–$10^{-9}$ M in young and $10^{-9}$ M in aged animals (Fig. 1B). In contrast to PTH [1–34], treatment of young and aged duodenum with the $NH_2$-terminal-shortened fragment of the hormone, PTH [7–34], was ineffective in increasing [3H]AA release above basal levels (data not shown). We then examined the effects of the intracellular $Ca^{2+}$ chelator 1,2-bis(O-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid, sodium (BAPTA-AM, 5 μM), and the extracellular $Ca^{2+}$ chelator EGTA (0.5 mM) on hormone-induced [3H]AA release. As shown in Figure 2A, BAPTA-AM fully suppressed PTH-dependent AA production in young enterocytes and inhibited by 50% PTH effects in cells from old animals, suggesting that hormone-dependent AA release in young animals occurs mainly through a $Ca^{2+}$-dependent mechanism, while in aged rats AA release dependent on PTH occurs through both a $Ca^{2+}$-dependent and -independent mechanism. EGTA did not alter the effects of PTH on AA release in both, young and old animals, indicating that the influx of extracellular calcium is not required for PTH-induced AA release in intestinal cells (Fig. 2B). To further establish the involvement of the $Ca^{2+}$-independent PLA2 (iPLA2) in aged animals, we measured [3H]AA release in the presence of the iPLA2 inhibitor palmitoyl trifluoromethyl ketone (PACOCF$_3$). Preincubation with 8 μM PACOCF$_3$, did not modify PTH response in young animals, but inhibited by 50% PTH-dependent AA release in old rats, confirming
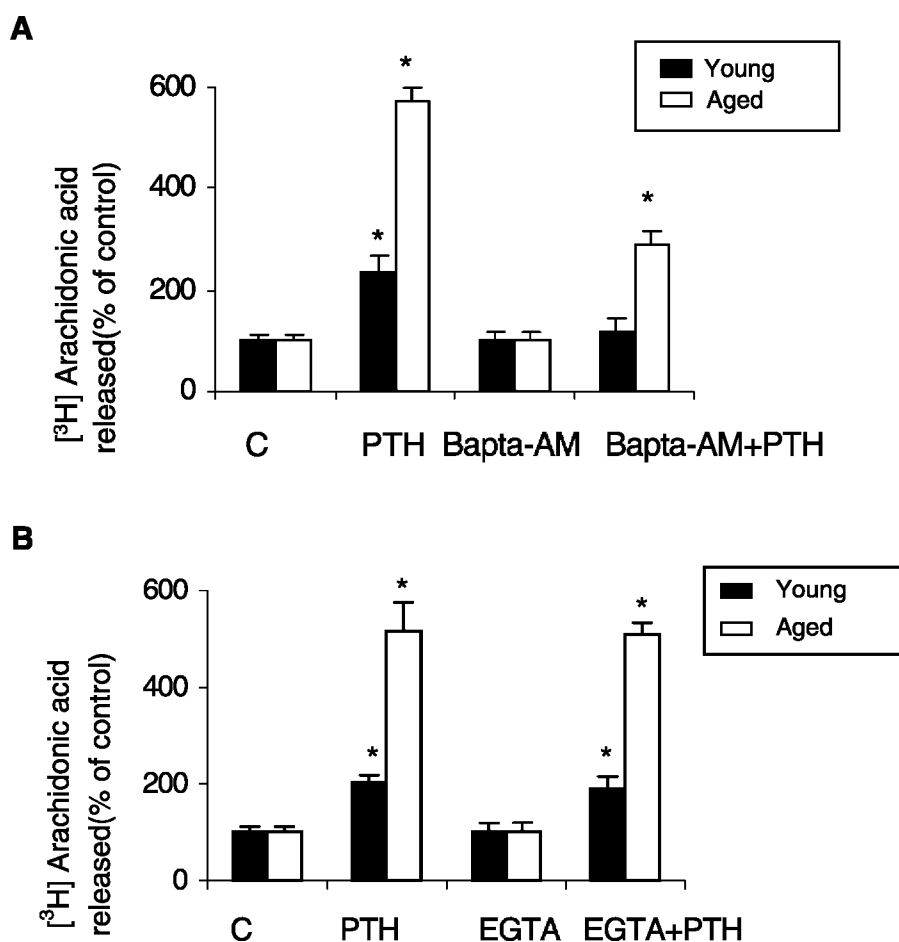


**Fig. 2.** Effect of BAPTA-AM and EGTA on [3H] AA release dependent on PTH. Release of [3H]AA was measured with or without BAPTA-AM (5 μM) (**A**) or EGTA (0.5 mM) (**B**), for 15 min in the presence or absence of PTH ($10^{-8}$ M). Data represent the average of three independent experiments performed in quadruplicate. Mean ± SD are given. *$P < 0.025$ with respect to its corresponding control.
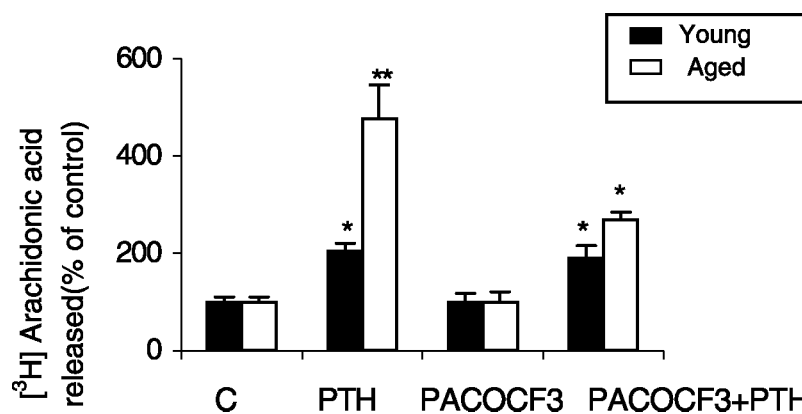
**Fig. 3.** Effect of PACOF$_3$ on [$^3$H] AA release dependent on PTH. Release of [$^3$H]AA was measured with or without PACOF3 (8 μM), for 15 min in the presence or absence of PTH (10$^{-8}$ M). Data represent the average of three independent experiments performed in quadruplicate. Mean ± SD are given. *$P < 0.025$, *$P < 0.05$ with respect to its corresponding control.

the activation of both, iPLA2 and cPLA2 by the hormone with aging (Fig. 3).

Activation of cPLA2 is tightly controlled by cytoplasmic Ca$^{2+}$ levels and by phosphorylation following various stimuli [Kudo and Murakami, 2002]. To evaluate PTH effects on cPLA2 phosphorylation, the cells were stimulated with PTH and 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 Figure 4 show, that PTH exposure of young enterocytes increased the level of serine phosphorylation of cPLA2, peaked at 10 min (+7-fold) and remained higher after 25 min exposure, with similar kinetics to those found for the release of AA (Fig. 1A). Basal levels of cPLA2 serine-phosphorylation were higher in old enterocytes, affecting hormone effects which were greatly diminished, increasing only 2- to 3-fold at 10 min and reaching values near basal after 30 min of PTH stimulation. The possibility that an increase in cPLA2 expression levels upon aging may contribute to these differences was excluded, as immublot analysis using an anti-cPLA2 antibody showed equal amounts of cPLA2 protein in unstimulated enterocytes from young and old animals (Fig. 5). As shown in Figure 6, PTH-dependent cPLA2 serine-phosphorylation, in both, young and aged animals, was totally suppressed by chelation of intracellular calcium with 5 μM BAPTA-AM, confirming the Ca$^{2+}$dependence of cytosolic PLA2.

We have previously reported that PTH activates MAPK (ERK1 and ERK2) in rat intestinal cells [Gentili and Russo de Boland, 2000]. Therefore, we explore the possibility that this signaling pathway may contribute to PTH effects on cPLA2. To that end, we studied the role of ERKs in mediating PTH serine-phosphorylation of cPLA2 and [$^3$H]AA release, and analysed the effect of the specific MEK inhibitor PD-98059. Preincubation of enterocytes with 20 μM PD-98059 inhibited to a great extent both PTH-induced cPLA2 phosphorylation (Fig. 6) and [$^3$H]AA release (Fig. 7) in both, young and aged animals, and thus suggest a role of the MAPK pathway in the activation of enterocyte cPLA2 by PTH.

Previous investigations have shown that PTH markedly increases the activity of adenylyl cyclase and cAMP levels (approximately 190 and 170%, respectively) [Picotto et al., 1997] and also increases PKC activity in rat enterocytes [Massheimer et al., 2000]. We tested whether these two pathways are also involved in the serine phosphorylation of cPLA2. To that end, enterocytes were preincubated with Rp-cAMP (0.2 mM), a highly specific competitive antagonist for all activators of the cAMP signal pathway [Botelho et al., 1988], or the PKC inhibitor Ro31820 (200 nM), followed by hormone stimulation. Both, the cAMP antagonist and the PKC inhibitor suppressed, to a great extent (50 and 60%, respectively), hormone-dependent cPLA2 phosphorylation in young enterocytes, being both compounds less effective in aged animals (40 and 30%, respectively) (Fig. 8).

The Ca$^{2+}$-dependent translocation of cPLA2 from the cytosol to intracellular membranous compartments rather than to the plasma

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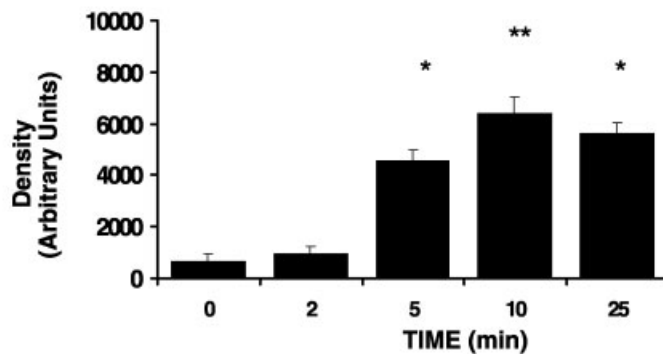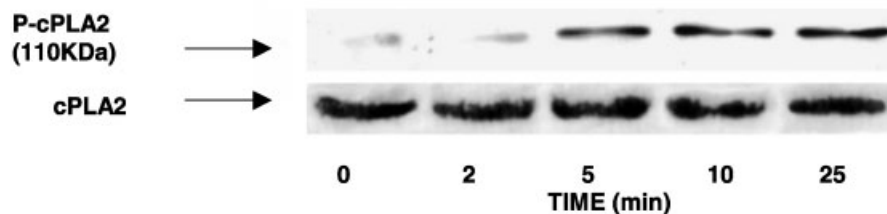




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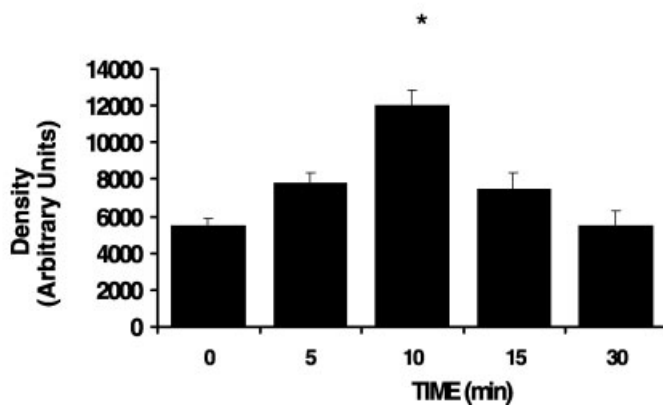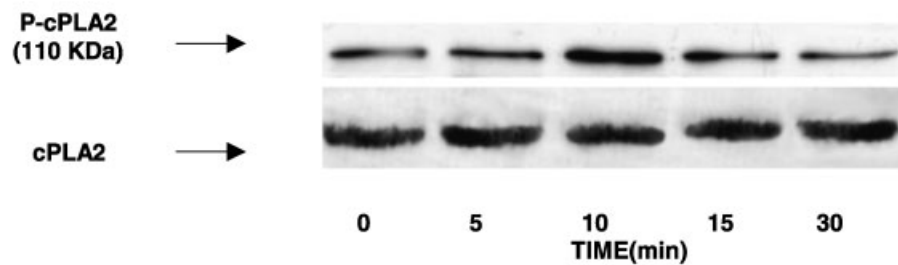




**Fig. 4.** cPLA2 is phosphorylated on serine 505 after stimulation of rat enterocytes with PTH. Enterocytes isolated from young (3 months) and aged (24 months) rats were treated with PTH ($10^{-8}$ M) for the indicated times. Upon reaction termination, cells were sonicated and soluble fractions were 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. Representative images and bar graphs of phospho cPLA2 quantified by scanning densitometry of blots from three independent experiments are shown. $*P < 0.025$, $**P < 0.01$ with respect to the corresponding control.
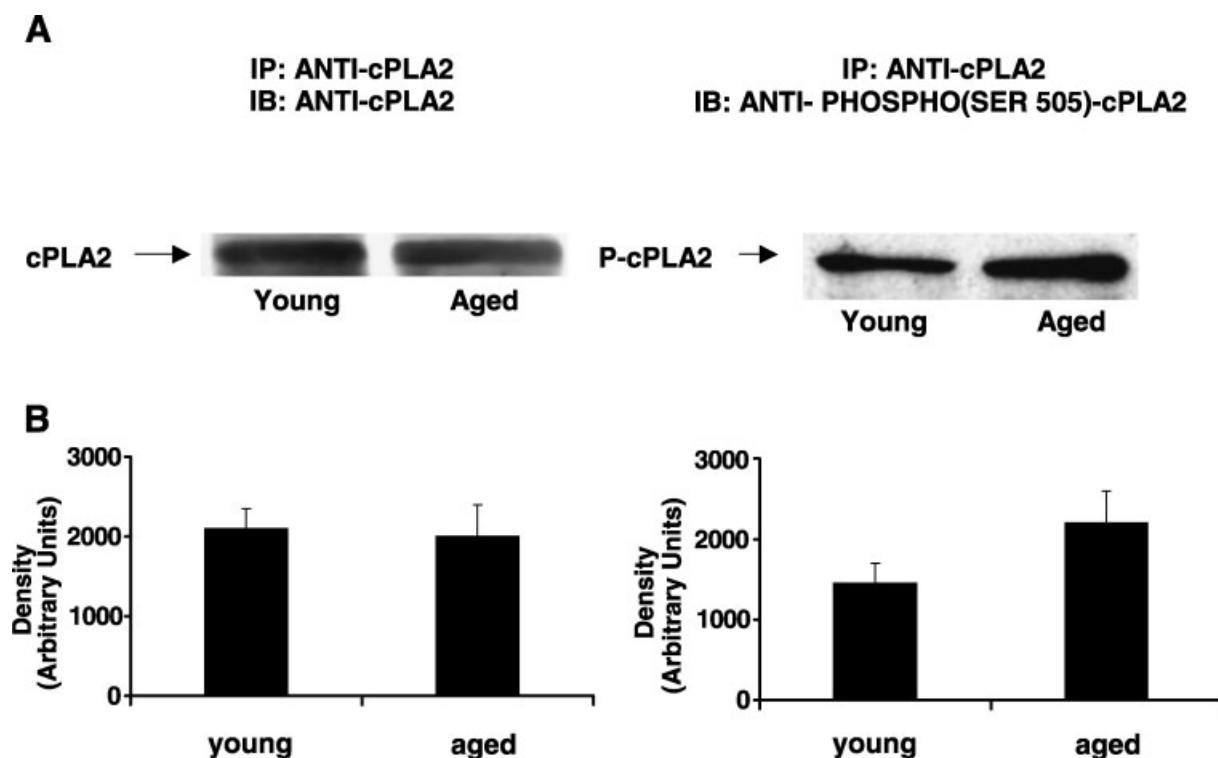
**A**



**B**



**Fig. 5.** Levels of cPLA2 and phospho-cPLA2 in enterocytes from young and old rats. Cell lysates of enterocytes isolated from young (3 months) and aged (24 months) were analyzed by SDS–PAGE followed by immunoblotting with anti-cPLA2 and anti P-cPLA2 as described under "Materials and Methods." **A**: Representative immunoblots (**B**) quantification of blots from three independent experiments is shown.

membrane is essential for the initiation of AA release [Schievella et al., 1995]. Therefore, the location of phosphorylated cPLA2 in cytosol and membrane fractions in resting and in enterocytes stimulated by PTH was studied by immunoblot analysis. To that end, cells were stimulated with the 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 Figure 9, phospho-cPLA2 was poorly detected in the cytosol of resting cells. On activation with PTH, phospho-cPLA2 appears within 10 min in the nuclei and microsomal membranes of young enterocytes. With aging, phopho-cPLA2 translocates to the membrane fraction, remaining part of the active enzyme in the cytosol.

## DISCUSSION

Aging is associated with increased circulating PTH levels [Chan et al., 1992] and decreased serum vitamin D metabolites [Armbrecht et al.,

1984], intestinal calcium absorption [Horst et al., 1978], and bone density [Hui et al., 1988]. Also, an age-related decline in PTH-stimulated AC activity in both rat kidney slices and cell membranes [Armbrecht et al., 1986; Hanai et al., 1989] has been shown. Upon aging, reduced PTH stimulation of cAMP levels [Egrise et al., 1992; Parfitt et al., 1992] or an increase in hormone-dependent cAMP accumulation [Pfeilschifter et al., 1993] has been observed in bone cells. In rat duodenal cells, we have obtained evidence of alterations in PTH-signaling systems with aging. The hormone increased enterocyte $^{45}Ca^{2+}$ influx, the absolute levels of cAMP and AC activity to a greater extent in aged than in young rats [Massheimer et al., 2000], whereas the early production of $IP_3$ and DAG generated by PTH was blunted in old animals. In enterocytes from aged rats, the hormone induced c-Src tyrosine dephosphorylation, a major mechanism of c-Src activation [Piwnica-Worms et al., 1987; Brown and Cooper, 1996], was also blunted and PLCγ phosphorylation via the non-receptor tyrosine kinase c-Src was impaired [Gentili et al., 2000].
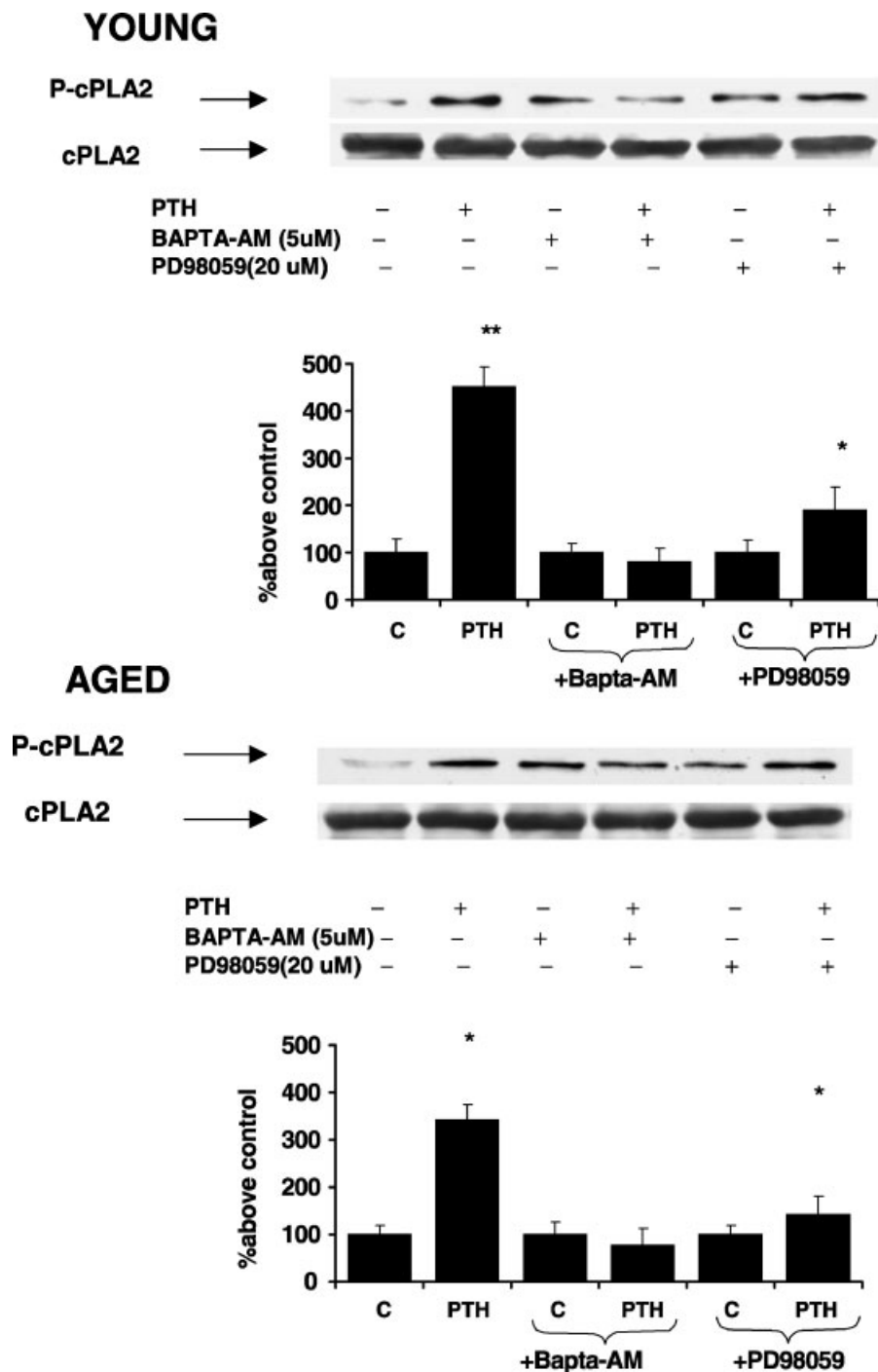
**Fig. 6.** Effect of BAPTA-AM and MAP kinase inhibitor PD 98059 on PTH-induced cPLA2 serine-phosphorylation. Enterocytes isolated from young (3 months) and aged (24 months) rats were treated with BAPTA-AM (5 μM) or PD98059 (20 μM) before stimulation by PTH ($10^{-8}$ M) for 10 min. Lysates were immunoblotted with anti-phospho serine 505 cPLA2 antibody. The immunoblots were stripped and re-probed with anti-cPLA2 antibody. Representative images and bar graphs of phospho cPLA2 quantified by scanning densitometry of blots from three independent experiments are shown. $*P < 0.025$, $**P < 0.01$ with respect to the corresponding control.

Although the relative levels of p42 and p44 MAPK did not change with age, the magnitude of PTH-dependent MAPK phosphorylation was significantly lower in enterocytes of aged rats compared with those of young animals [Gentili and Russo de Boland, 2000]. In this study, we analyzed for the first time, alterations in PLA2 activity and response to PTH in rat enterocytes
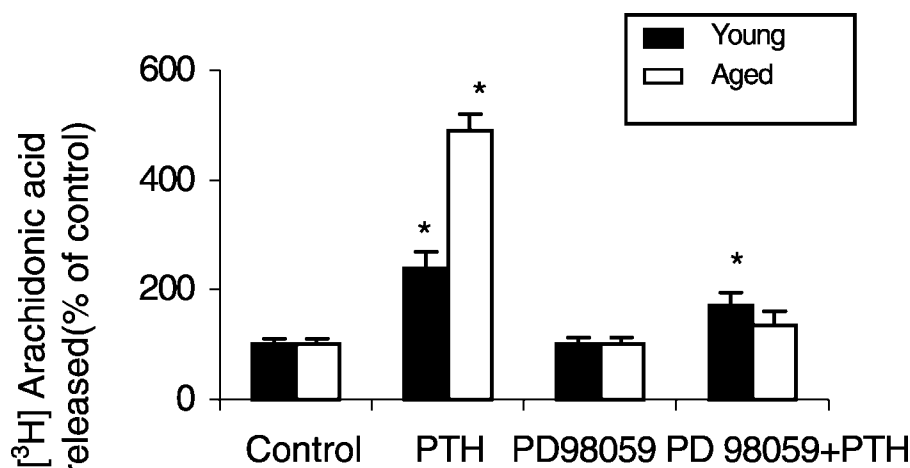
**Fig. 7.** Effect of MAPK inhibitor PD-98059 on [³H] AA release dependent on PTH. Release of [³H]AA was measured with or without PD-98059 (20 μM), for 15 min in the presence or absence of PTH (10⁻⁸ M), as described in the Figure 1. Data represent the average of two independent experiments performed in quadruplicate. Mean ± SD are given. *$P < 0.025$ with respect to its corresponding control.

with aging. We found that PTH, rapidly stimulate AA release in rat duodenal cells, effect that is greatly potentiated by aging. We also found that hormone-induced AA release is $Ca^{2+}$-dependent in young animals, while half of total AA released by PTH in cells from aged rats is $Ca^{2+}$-independent and that $Ca^{2+}$ release from internal stores is both necessary and sufficient for the activation of calcium-dependent PLA2 during PTH-mediated mobilization of AA in intestinal cells. This polyunsaturated fatty acid, is released in response to a large number of bioactive molecules and is involved in the mediation of several important biological functions including vascular contraction/relaxation, cell proliferation/differentiation, and cell survival/apoptosis [Glasgow et al., 1992; Dethlefsen et al., 1994; Gronich et al., 1994; Rao et al., 1994; Goetzl et al., 1995; Balsinde and Dennis, 1996; Leslie, 1997; Pfister et al., 1998; Chen et al., 2001; Panini et al., 2001]. PLA2s are the major rate-limiting enzymes in the release of AA in many cell types [Balsinde and Dennis, 1996; Leslie, 1997; Pontus et al., 1998]. Among the growing number of PLA2s that have been isolated and characterized thus far, a calcium-dependent high molecular mass cytosolic PLA2 (cPLA2) and a calcium-independent PLA2 (iPLA2) have been shown to play an important role in AA release in response to a number of stimulants including receptor tyrosine kinase (RTK) and G protein-coupled receptor (GPCR) agonists [Lin et al., 1992; Pontus et al., 1998; Shinohara et al., 1999; Gijon et al., 2000;

Jenkins et al., 2002]. An interesting finding of our work is that, cPLA2 and the $Ca^{2+}$-independent iPLA2, potentiate PTH-induced AA release in aged enterocytes.

The necessity of both iPLA2 and cPLA2 in AA liberation in aged animals, could possibly be due to a sequential interplay between the enzymes leading to maximal AA release as reported with sPLA2 and cPLA2 in response to inflammatory stimuli in other cells [Balsinde and Dennis, 1996; Anthonsen et al., 2001]. The activity of iPLA2 is $Ca^{2+}$-independent, but its detailed substrate specificity and enzymology remain to be elucidated. Each splice variant of iPLA2 shows a unique tissue distribution, yet in general terms this enzyme is ubiquitously expressed in a wide variety of cells and tissues [Ackermann and Dennis, 1995], including in rat small intestine [Fukushima and Serrero, 1994].

Cellular cPLA2 activities are tightly regulated by different factors, including $Ca^{2+}$ and phosphorylation [Clark et al., 1995; Leslie, 1997; Hirabayashi and Shimizu, 2000]. It has been well established that calcium binds to the amino-terminal C2 domain [Nalefski et al., 1994] and drives its membrane binding in vitro [Nalefski et al., 1997; Nalefski et al., 1998; Bittova et al., 1999] and in the cell [Gijon et al., 1999; Perisic et al., 1999]. Phosphorylation has also been shown to activate cPLA2, but this regulatory mechanism appears to be complex. It was initially found that the phosphorylation of Ser 505 by mitogen-activated protein kinases
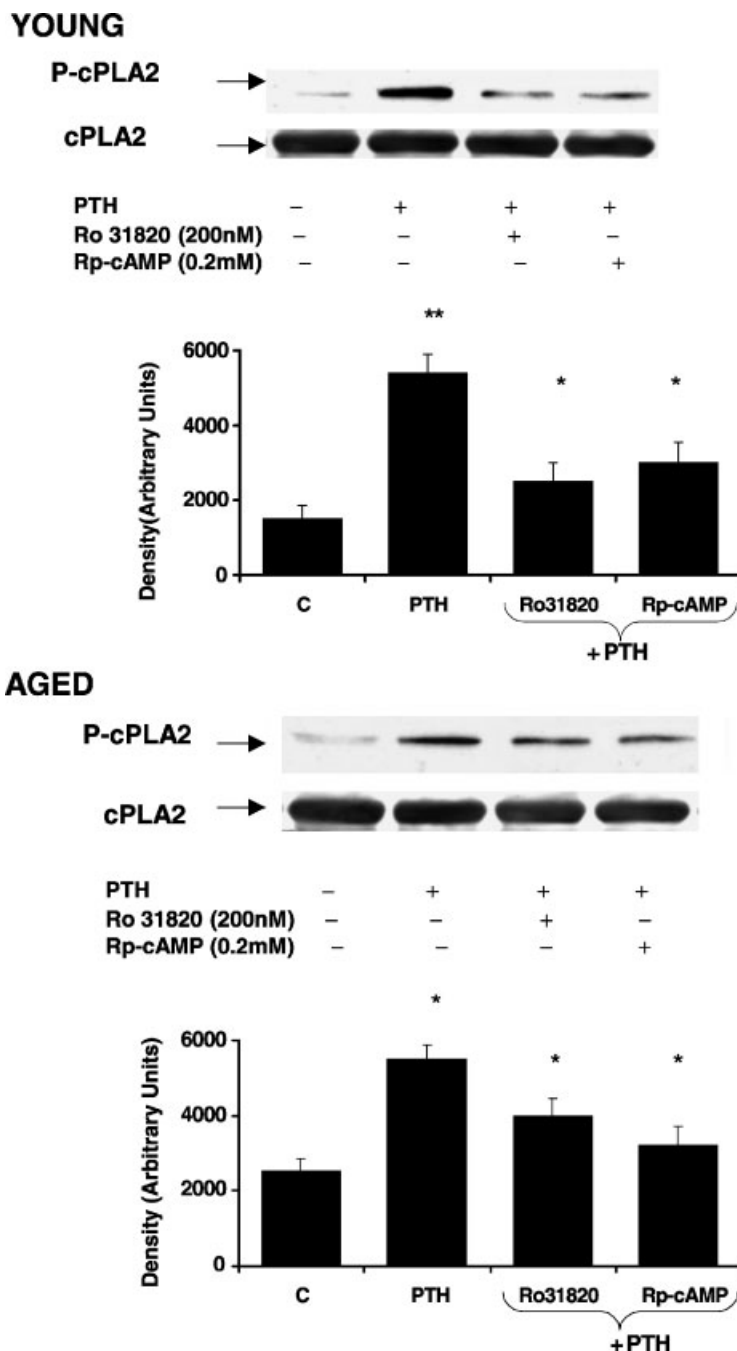
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**Fig. 8.** Rp-cAMP and Ro31820 partially suppressed PTH-induced cPLA2 serine-phosphorylation. Enterocytes isolated from young (3 months) and aged (24 months) rats were treated with PTH ($10^{-8}$ M) for 10 min in the presence or absence of the cAMP antagonist, Rp-cAMP (0.2 mM) or the PKC inhibitor, Ro31820 (200 nM). 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. Representative images and bar graphs of phospho cPLA2 quantified by scanning densitometry of blots from three independent experiments are shown. $*P < 0.025, **P < 0.01$ with respect to the corresponding control.

is essential for agonist-induced AA release in Chinese hamster ovary cells [Lin et al., 1993]. Further studies have shown, however, that the effect of Ser 505 phosphorylation on agonist-induced AA release is cell type- and agonist-dependent. For instance, Ser 505 phosphorylation of cPLA2 is not required for the AA release in thrombin-stimulated platelets [Kramer et al.,
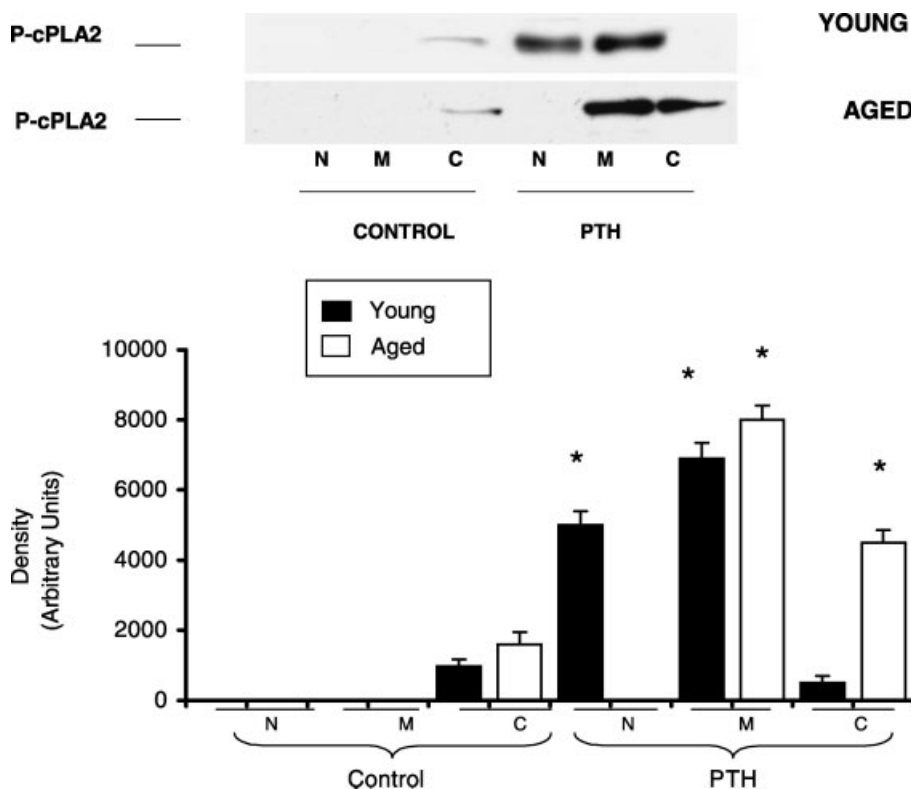
**Fig. 9.** cPLA2 translocates to enterocyte microsomal membranes and nuclei in response to PTH. After treatment with PTH ($10^{-8}$ M, 10 min), cells were homogenized and separated into cytosol, nuclei, and membrane fractions by centrifugation, as described in "Materials and Methods." Equal portions of each fraction were subjected to SDS–PAGE and immunoblotted with anti-phospho serine 505 cPLA2 antibody. Results are the average of three independent experiments ± SD. *$P < 0.05$ with respect to the corresponding control.

1996]. In macrophages, Ser 505 phosphorylation is not essential for AA release in response to agonists that induce a sustained increase in calcium, but appears to be important when there is a transient increase in calcium [Qiu et al., 1998]. In enterocytes from 3- and 24-months-old rats, PTH induced, in a time and dose-dependent fashion, the phosphorylation of cPLA2 on serine 505, effect that was totally suppressed by chelation of intracellular calcium with BAPTA-AM. Biophysical and cell studies of wild type and phosphorylation site mutants reveal that the Ser 505 phosphorylation activates cPLA2 by significantly slowing the dissociation of membrane-bound cPLA2, thereby improving its overall membrane binding affinity [Das et al., 2003]. Basal levels of cPLA2 serine-phosphorylation were higher in old enterocytes, affecting the hormone response which was greatly diminished. cPLA2 phosphorylation impairment in old animals was not related to changes in cPLA2 protein expression and did not explain the substantial increase on

PTH-induced AA release with aging, further suggesting the involvement of a different PLA2 isoform. While there are several sites on cPLA2 that can be phosphorylated [Leslie, 1997], it is generally agreed that cPLA2 can be phosphorylated on serine 505 by ERK2 in vitro. However, there is strong evidence suggesting that kinases other than ERK1/2 may be involved in the phosphorylation of cPLA2 [Qiu and Leslie, 1994; Borsch-Haubold et al., 1995]. Kinases such as PKC and PKA can phosphorylate cPLA2 in vitro [Leslie, 1997] and more recently, it was reported that phosphorylation of Ser 515 by calcium-/calmodulin-dependent protein kinase II in vascular smooth muscle cells increased the enzymatic activity of cPLA2 [Muthalif et al., 2001]. Finally, it has been suggested that phosphatidylinositol 4,5-bisphosphate can specifically activate cPLA2 [Mosior et al., 1998; Das and Cho, 2002] by inducing conformational changes of the enzyme and thereby juxtaposing its active site and the membrane surface [Das and Cho, 2002]. The data presented here show

several new interrelated points concerning the
kinases that phosphorylate cPLA2 in rat intest-
inal cells under the effects of PTH. We found
that several kinases are involved in cPLA2
phosphorylation, these include ERK 1/2, PKC,
and PKA. We have previously shown that PKA
activation is upstream of ERK1/2 activation
[Gentili et al., 2001], and the fact that PTH-
induced phosphorylation of cPLA2 is sup-
pressed to a great extent by cAMP antagonists,
suggest that the effect of PKA on cPLA2 is
mediated through ERK1/2. Upon activation,
cPLA2 translocates from the cytosol to intra-
cellular membranous compartments, allowing
access to the arachidonoyl-containing phospho-
lipid substrate [Schievella et al., 1995]. We show
that in rat enterocytes, PTH-dependent trans-
location of phosphorylated cPLA2 from the
soluble to membrane fractions, is modified by
aging, where in contrast to young animals, a
great proportion of phospho-cPLA2 remained
cytosolic.

In conclusion, the results of this study
demonstrate that PTH stimulates the rapid
release of AA from rat enterocytes and that the
ERK1/2 pathway, PKA, PKC and increases in
intracellular calcium act in concert to stimulate
the cPLA2 enzyme, resulting in the liberation of
AA. We show for the first time, that aging alters
the release of AA by PTH in intestinal cells,
cPLA2 is the major enzyme responsible for AA
release in young enterocytes while cPLA2 and
the $Ca^{2+}$-independent iPLA2, potentiate PTH-
induced AA release in aged cells. Impairment of
PTH activation of PLA2 isoforms upon aging
may result in abnormal hormone regulation
of membrane fluidity and permeability and
thereby affecting intestinal cell membrane
function.

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