Protein kinase C inhibitors stimulate arachidonic and docosahexaenoic acids release from uterine stromal cells through a Ca^{2+}-independent pathway

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Abstract The mechanisms underlying arachidonic acid (AA) release by uterine stromal (U_{III}) cells were studied. Stimulation of AA release by calcium ionophore and PMA are inhibited by various PKC inhibitors and by calcium deprivation. These results suggest the involvement of an AA-specific $cPLA_2$ as the release of docosahexaenoic acid (DHA) from prelabelled cells is much lower than the release of AA. The results also show a more original stimulation of AA and DHA release induced by PKC inhibitors, which is insensitive to calcium deprivation. This stimulation is not due to acyltransferase inhibition, suggesting the participation of a Ca^{2+}-independent PLA_2 ($iPLA_2$). However, $iPLA_2$ activity measured in U_{III} cells is inhibited by the specific $iPLA_2$ inhibitor, BEL, and is not stimulated by PKC inhibitors, in contrast with the AA and DHA release. It seems therefore that this $iPLA_2$ cannot be involved in this mechanism. The participation of another $iPLA_2$, BEL-insensitive, is discussed.

Key words: Arachidonic and docosahexaenoic acids; Ca^{2+}-independent PLA_2; PKC inhibitor; Uterine stromal cell

1. Introduction

A key regulatory event in most cell types is the mobilization of esterified arachidonic acid (AA) from cellular stores. AA and its oxygenated metabolites (eicosanoids) then induce biochemical events which alter numerous cell functions. One of the major mechanisms involved in AA mobilization is the hydrolysis of membrane arachidonoyl-sn-2-phospholipids catalyzed by phospholipase A_2 [1,2]. We have established a uterine stromal cell line, named U_{III}, from adult rat [3] whose growth is regulated by polyunsaturated fatty acids (PUFA). Arachidonic acid significantly increases U_{III} cell growth whereas docosahexaenoic acid (DHA) at micromolar concentrations induces a dose-dependent inhibition of cell proliferation not related to the production of peroxidation products [4]. A number of other fatty acids could not elicit these effects showing the specificity of AA and DHA. Moreover, the proliferation effect could be blocked in a dose-dependent and reversible manner by inhibitors of phospholipase A_2 but not by inhibitors of eicosanoid synthesis [5], suggesting that the polyunsaturated fatty acid was directly responsible for this effect. This conclusion is supported by the work of others who have shown proliferation-dependent changes in release of arachidonic acid from endothelial cells [6] and fibroblasts [7]. AA release from phospholipids decreased with confluence, and phospholipase A_2 activity changed in concert with the alteration of the release.

There is now increasing evidence that a cytoplasmic phospholipase A_2 ($cPLA_2$), which specifically catalyses the release of AA from the sn-2 position of phosphoglycerides, could be the main pathway of arachidonate mobilization in activated cells [8–11]. Activation of the enzyme is thought to occur by two mechanisms. One mechanism involves a Ca^{2+}-dependent translocation of $cPLA_2$ from the soluble to membrane fractions of cells, allowing $cPLA_2$ access to its substrate [12]. The second involves phosphorylation of $cPLA_2$, resulting in stimulation of its intrinsic enzyme activity [13]. Mitogen-activated protein kinases (MAPK) have been proposed as the enzymes involved in $cPLA_2$ phosphorylation and PKC-dependent and PKC-independent mechanisms for the activation of MAPK, and consequently of $cPLA_2$, have been described [14].

In order to focus upon the role of PKC on the release of AA, we have assessed the effects of known inhibitors of protein kinases on the release of radioactive AA in prelabelled cells stimulated with the Ca^{2+}-ionophore A23187, to mimic the effects of a Ca^{2+}-mobilizing ligand or with PMA, a direct activator of PKC. The results show that the stimulation of AA release by A23187 and PMA stimulation was inhibited by PKC and tyrosine kinase inhibitors and by calcium deprivation. The results also show a more original stimulation of AA and DHA release induced by PKC inhibitors in resting cells, which is insensitive to calcium deprivation. This result is interesting as it involves a regulatory pathway different from the stimulation of AA release by $cPLA_2$. This pathway is not regulated by acyltransferases as shown by the absence of effect of calphostin C on [^{3}H]AA or [^{3}H]DHA incorporation into phospholipids. The involvement of Ca^{2+}-independent PLA_2 ($iPLA_2$) was also investigated. The $iPLA_2$ activity measured

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Abbreviations: PLA_2, phospholipase A_2; $cPLA_2$, cytosolic PLA_2; $iPLA_2$, Ca^{2+}-independent PLA_2; AA, arachidonic acid; DHA, docosahexaenoic acid; MAPK, mitogen-activated protein kinase; PKC, protein kinase C; PKA, protein kinase A; H-7, [1-(5-isoquinolinesulfonyl)-2-methyl piperazine, HCl]; H-89, {*N*-[2-((*p*-bromo-cinnamyl)amino)ethyl]-5-isoquinoline sulfonamide, HCl}; BEL, bromo*enol*lactone ((*E*)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2*H*-pyran-2-one); PMA, phorbol myristate acetate; EGTA, [ethylenebis (oxyethylenenitrilo)] tetraacetic acid; EDTA, (ethylenediaminetetraacetic acid, sodium); BAPTA, [1,2-bis(*o*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacteic acid, sodium]; BSA, bovine serum albumin; DTT, dithiothreitol; DPPC, 1,2-dipalmitoyl phosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine

Enzymes: Phospholipase A_2 (EC 3.1.1.4).

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in U_{III} cells was inhibited by BEL, a suicide inhibitor of the best characterized $iPLA_2$ [15–17], and was not stimulated by PKC inhibitors, in contrast with AA and DHA release. Moreover, preincubation of cells with BEL did not impair the stimulation of AA release induced by calphostin C, suggesting that the BEL-sensitive $iPLA_2$ is not involved in this releasing pathway.

2. Material and methods

2.1. Chemicals

Tissue culture medium M199, L-glutamine, penicillin, streptomycin, fetal calf serum (FCS) were obtained from GIBCO (Cergy-Pontoise, France). [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (210 Ci/mmol), [4,5-^{3}H]docosahexaenoic acid (58 Ci/mmol), L-α-dipalmitoyl-[2-palmitoyl-1-^{14}C]phosphatidylcholine (55.5 mCi/mmol) were obtained from New England Nuclear (Boston, MA). Standard free fatty acids and phospholipids, fatty acid-free bovine serum albumin (BSA) and calphostin C were from Sigma (St. Louis, MO). Calcium ionophore A23187, phorbol 12-myristate 13-acetate (PMA), H-7, H-89 and staurosporine were from Calbiochem (La Jolla, CA). The Ca^{2+}-independent PLA_2 inhibitor (*E*)-16-(bromomethylene)tetrahydro-3-(1 naphthalenyl)-2*H*-pyran-2-one (bromo*enol*lactone, BEL) was synthesized as described previously by Daniels et al. [18]. All other chemicals were of analytical grade.

2.2. Cell culture

For stock culture, U_{III} cells were grown in medium 199 supplemented with 10% FCS, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. They were incubated in 75 cm^2 Falcon plastic flasks in a 95% air-5% CO_2 humidified atmosphere at 37°C. The medium was changed every 48 h. Confluent cells were subcultured by incubation with 0.25% trypsin, centrifuged and seeded at 1:2 ratio. Cell viability, determined by the trypan blue exclusion method, was consistently greater than 95%.

2.3. Incorporation and release of arachidonic and docosahexaenoic acids

Confluent cells from stock culture were trypsinized, resuspended into medium 199 with 10% FCS and seeded at 1.5×10^5 cells/well in 12-well culture plates and incubated for 24 h. The medium was then removed and cells were incubated for 8 h in medium 199 with 1 mg/ml fatty acid-free BSA that contained 0.5 μCi/ml of [^{3}H]AA or [^{3}H]DHA. The labelling medium was aspirated and cells were washed twice with medium 199+0.1% BSA followed by another incubation for 16 h at 37°C in medium 199+10% FCS, without label. The medium was then aspirated and cells were washed again three times with medium 199+0.1% BSA and incubated for 5 to 60 min in 1 ml of the same medium at 37°C with or without agonists. The radioactivity released in the medium was measured by scintillation spectrometry and was normalized as the percentage of the total incorporated radioactivity. Total radioactivity was defined as the sum of the radioactivity released in the medium and the radioactivity remaining in cell lipids.

Experiments were also performed to determine the distribution of the label within lipid and phospholipid classes at the end of the labelling period. Total lipids from washed cells and from media were extracted according to the procedure of Bligh and Dyer [19]. The amount of radioactivity was determined by liquid scintillation spectrometry on an aliquot of the organic phase. The distribution of radioactivity within lipid and phospholipid classes was determined after TLC and HPLC separation as described previously [20]. Total lipids were also submitted to transmethylation, the resulting fatty acid methyl esters (FAMEs) were separated by reversed phase HPLC [20] and their radioactivity was determined using a Radiomatic Flow One β detector.

2.4. Ca^{2+}-independent phospholipase A_2 assay

U_{III} cells were adjusted to a concentration of 1.2×10^7 cells/ml with homogenization buffer (10 mM HEPES, 1 mM EDTA, 1 mM DTT and 0.34 M sucrose), briefly sonicated and immediately centrifuged at $1000\times g$, 4°C for 5 min. The resulting low speed supernatant (LSS) was used for $iPLA_2$ assay, according to Ackermann et al. [21] with minor modifications. The standard assay contained 1.2 mM Triton X-100, 120 μM dipalmitoyl phosphatidylcholine (DPPC), 5 mM EDTA, 100 mM HEPES (pH 7.5) and 0.8 mM ATP in a final volume of 500 μl. The substrate was prepared by evaporating the required amounts of dipalmitoyl-PC- and 1-palmitoyl-2-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine (200 000 dpm per assay) to dryness under a stream of N_2. The dried phospholipids were resuspended with 450 μl of assay buffer. Mixed micelles were formed by a combination of heating (above 40°C), vortexing and water bath sonication until clarification.

Assays were initiated by the enzyme addition to the substrate mixture and were incubated at 40°C for 60 min with agitation. The reaction was stopped by the addition of 2 ml of chloroform-methanol (2:1, v/v) containing 0.02% butylated hydroxy toluene (BHT) as an antioxidant and 100 μg of oleic acid as a carrier. The extraction of lipids was completed by the addition of 0.2 ml of 2 mol/l KCl plus 0.5 mmol/l EDTA. After centrifugation ($900\times g$ for 5 min), the chloroform layer was removed by aspiration. A second extraction with 1 ml of chloroform was done and pooled chloroform extracts were evaporated to dryness under vacuum. Lipid classes were separated using anion exchange chromatography columns as described previously [22]. The $iPLA_2$ activity was determined from the radioactivity found in the unesterified fatty acid fraction. $iPLA_2$ activity values, measured in the LSS of U_{III} cells homogenate, were normalized to the protein content of the assay and expressed as pmol palmitic acid released min^{-1} mg^{-1} protein.

3. Results and discussion

3.1. [^{3}H]AA and [^{3}H]DHA labelling and release from phospholipids

TLC analysis of cellular lipids after labelling of the cells showed that more than 97% of the label was associated with phospholipids, only trace amounts were found in triglycerides and free fatty acids. HPLC analysis of cellular phospholipids demonstrated a distribution of the label, either [^{3}H]AA or [^{3}H]DHA, very close to the distribution of the corresponding endogenous fatty acid as measured by GLC analysis [20]. As expected, the distribution of the label within phospholipid classes was very different for [^{3}H]AA (PE, 38.4 ± 2.0%; PC, 28.5 ± 1.8%; PI, 25.6 ± 1.3%; PS, 1.3 ± 0.5%) and [^{3}H]DHA (PE, 67.0 ± 2.3%; PC, 14.1 ± 1.0%; PS, 5.5 ± 0.4% and PI ≤ 0.2%).

TLC analysis of lipids from media obtained from control and stimulated cells showed that more than 95% of the radioactivity released during the experiment was recovered as free fatty acids, and 1 to 3% were recovered as phospholipids. The latter likely correspond to the very few cells that detach from the monolayer. Only trace amounts of labelled triglycerides and sterol esters were found. No oxygenated derivatives from AA or DHA were observed in the medium. Reversed phase HPLC analysis of FAMEs from media showed that the radioactive fatty acid released was mainly AA (more than 90%) and its elongated derivative (22:4*n*−6, 4 to 8%) when cells were labelled with [^{3}H]AA and exclusively DHA when cells were labelled with [^{3}H]DHA (not shown).

3.2. Regulation of AA release in resting and A23187-activated cells

The incubation of cells with increasing concentrations of A23187 (0.1–1 μM) resulted in a dose-dependent increase of [^{3}H]AA release (Fig. 1). Short term treatment of cells with PMA alone, a direct activator of protein kinase C, did not induce any AA release (Fig. 1, insert). However, pretreatment with PMA (5 min) followed by A23187 resulted in a significantly larger increase in arachidonate release, compared to A23187-treated cells (Fig. 1, insert). This priming effect of

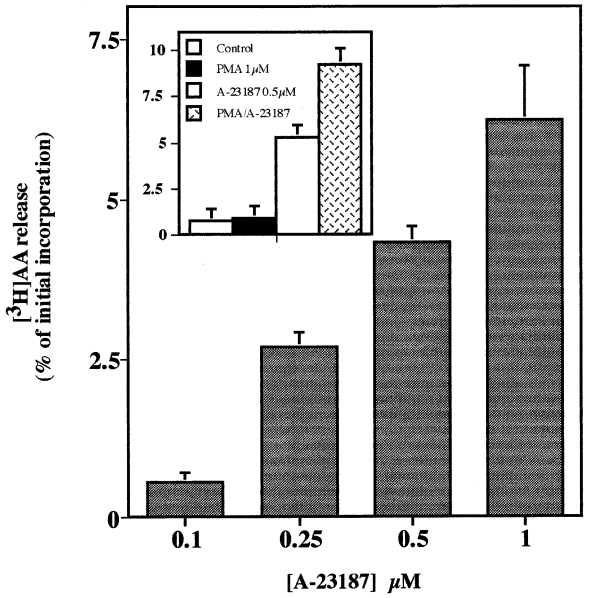

Fig. 1. Concentration-dependent release of [^{3}H]arachidonic acid by A23187-activated cells. Cells were labelled with [^{3}H]arachidonic acid, washed, and then incubated with the indicated concentration of A23187 for 15 min. The [^{3}H]AA released in the medium during this period of time was counted. The data are presented as the percentage of the total ^{3}H radioactivity initially incorporated in cells and expressed as means ± S.D. of three separate experiments. Insert: Cells labelled with [^{3}H]AA were washed and then incubated with medium only (control), 1 μM PMA for 15 min, 1 μM A23187 for 10 min, or 1 μM PMA for 5 min followed by 1 μM A23187 for 10 min (PMA/A23187). [^{3}H]AA released in the medium was measured. Results are means ± S.D. of three separate experiments.

PMA has been shown in a variety of cells [13,23] where phosphorylation of $cPLA_2$ must be combined with a calcium-mobilizing agonist to increase AA release. In other cell models, PMA can induce an increase in phosphorylation and catalytic activity of $cPLA_2$ as well as arachidonic release without an increase in intracellular calcium [24,25]. This suggests that cell-specific pathways for regulation of $cPLA_2$ activation and AA release may exist.

To determine the importance of phosphorylation in A23187-induced AA release, the effect of different protein kinase inhibitors was examined. Pretreatment of U_{III} cells with H-89, a potent and selective inhibitor of PKA, was without effect on both basal and A23187-induced arachidonate release (Fig. 2). The tyrosine kinase inhibitor genistein has no effect on the basal release of [^{3}H]AA but inhibited the arachidonate release induced by A23187 by 53% ($P < 0.01$) (Fig. 2). The protein kinase C inhibitors, H-7, staurosporine and calphostin C, also inhibited the release of [^{3}H]AA in cells stimulated by A23187 (38%, $P < 0.01$, 52%, $P < 0.01$ and 65%, $P < 0.01$, respectively) (Fig. 2b). They also completely blocked the priming effect of PMA on A23187-induced AA release (not shown), as previously shown by Lin et al. [13] with staurosporin in CHO cells. Surprisingly, these PKC inhibitors significantly increased the release of [^{3}H]AA in non-stimulated cells by 63%, 45% and 112%, respectively (Fig. 2a). This unexpected stimulation of basal AA release was dose-dependent up to 250 nM when studied in response to calphostin C (Fig. 3).

The differences in the effects of PKC inhibitors in resting and stimulated U_{III} cells suggest that different pathways for AA release may occur, depending on cell activity. In order to test this hypothesis, we compared the effect of calphostin C in resting and A23187-activated cells labelled either with [^{3}H]AA or [^{3}H]DHA, the latter being poorly released by $cPLA_2$ compared to AA [26]. We also tested the dependency of calphostin C-induced fatty acid release on calcium. Results are summarized in Fig. 4. Unstimulated cells, prelabelled with [^{3}H]AA or [^{3}H]DHA, released similar amounts of these radioactive fatty acids in the culture medium. Calphostin C increased the release of [^{3}H]AA and [^{3}H]DHA to the same extent (about 2-fold). By contrast, A23187 increased much more the release of [^{3}H]AA (about 7-fold) than that of [^{3}H]DHA (about 2-fold). Moreover, the chelation of extracellular Ca^{2+} by EGTA (3 mM), which prevents the influx of Ca^{2+}, had no effect on

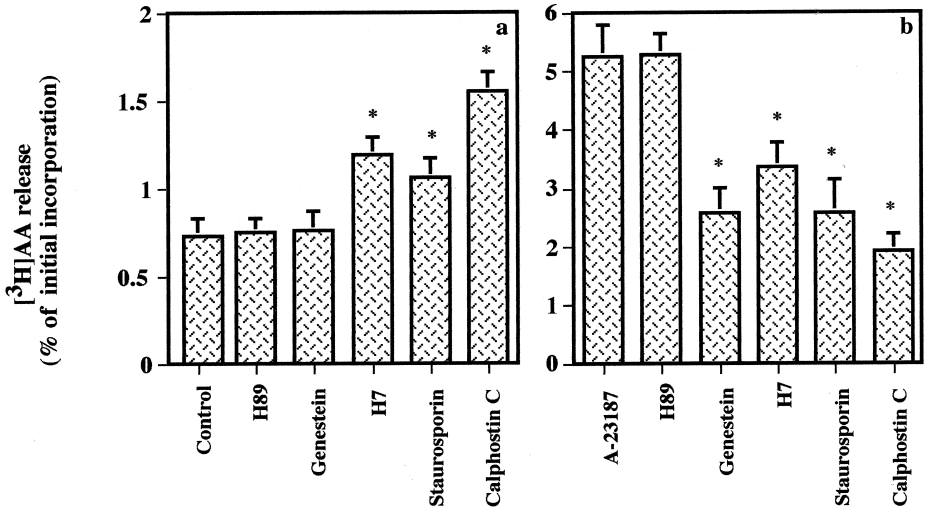

Fig. 2. Effect of protein kinase inhibitors on the basal (a) or A23187-stimulated (b) release of [^{3}H]AA from U_{III} cells. Cells labelled with [^{3}H]AA were preincubated for 45 min with or without (control) 1 μM H-89, 370 μM genistein, 200 μM H-7, 500 nM staurosporin, or 250 nM calphostin C, washed and then incubated with medium only (a), or 0.5 μM A23187 for 15 min (b). [^{3}H]AA released in the medium was measured. Data, expressed as the percentage of total radioactivity initially incorporated in cells, are means ± S.D. of three separate experiments. *Significantly different ($P < 0.01$) from the corresponding control.

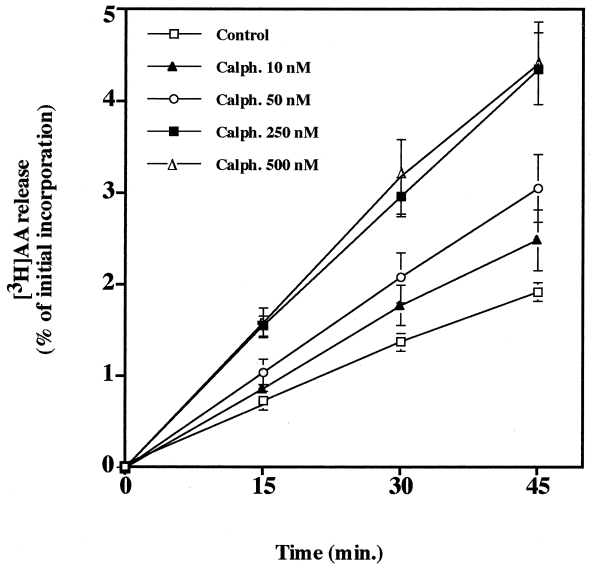

Fig. 3. Concentration-dependent release of [^{3}H]AA by U_{III} cells pretreated with calphostin C. Cells labelled with [^{3}H]AA were preincubated for 45 min with or without (control) calphostin C at various concentrations and washed before the [^{3}H]AA released in the medium for 15, 30 or 45 min was measured. Data are expressed as the percentage of the total radioactivity initially incorporated in cells and expressed as means ± S.D. of three independent measurements.

calphostin C-induced AA or DHA release whereas the stimulatory effect of A23187 was completely abolished. The same results were observed when BAPTA (100 μM), an intracellular Ca^{2+} chelator, was used in addition with EGTA (not shown).

These data show that the regulation of arachidonate mobilization within U_{III} cells could occur at least by two different pathways. The stimulation of AA release by calcium ionophore and PMA, which is inhibited by various PKC and tyrosine kinase inhibitors and calcium deprivation, is in accordance with previously reported data in other cell types [9,13,23,27] where the regulation of $cPLA_2$ in terms of phosphorylation and Ca^{2+}-dependent activation has been well characterized [14,28]. The results suggest the involvement of an AA-specific $cPLA_2$, as the release of DHA from prelabelled cells was much lower than the release of AA. The data also demonstrate a more original stimulation of basal AA release induced by PKC inhibitors. A similar finding has been reported in human neutrophils [29] and more recently in human platelets [30] where calphostin C, but not staurosporin and H-7, also induced a direct release of AA. This regulatory pathway is insensitive to calcium deprivation and equally affects DHA release, suggesting that it is different from the stimulation of AA release by $cPLA_2$. Moreover, the absence of effect of genistein, which strongly reduces the A23187-induced AA release, and of H-89, suggests that this pathway in not regulated by tyrosine kinases nor by cAMP.

3.3. Role of acyltransferases

Increase of AA release could result from the activation of a Ca^{2+}-independent PLA_2 or from the inhibition of acyltransferases. There have been several reports concerning the regulation of acyl-CoA: lysophospholipid acyltransferase activity by PKC. Treatment of macrophages and platelets with activators of PKC inhibits arachidonoyl CoA acyltransferase which results in an increase in AA release and a decrease in AA uptake by the cells [31,32]. In order to assess the role of acyltransferases, we studied the incorporation of exogenous [^{3}H]AA and [^{3}H]DHA into cellular phospholipids in the absence or presence of calphostin C. Merthiolate, a known inhibitor of acyltransferases [33] was used as control. Cells were pretreated with 250 nM calphostin C or merthiolate (25–100 μM) and then incubated with [^{3}H]AA or [^{3}H]DHA for 30 min in the presence of calphostin C or merthiolate. The radioactivity incorporated in phospholipids was measured at the end of the incubation as described in Section 2. Treatment of cells with calphostin C did not induce any significant modification of AA or DHA incorporation into phospholipids. Merthiolate, as expected, led to a decrease in AA uptake by the cells (19%, 33% and 44% with 25, 50 and 100 μM merthiolate after 30 min incubation, respectively), and to an increase by 2-fold, 4-fold and 10-fold in AA release from prelabelled cells when incubated with 25, 50 and 100 μM merthiolate, respectively, for 30 min. The lack of any inhibitory effect of calphostin C,

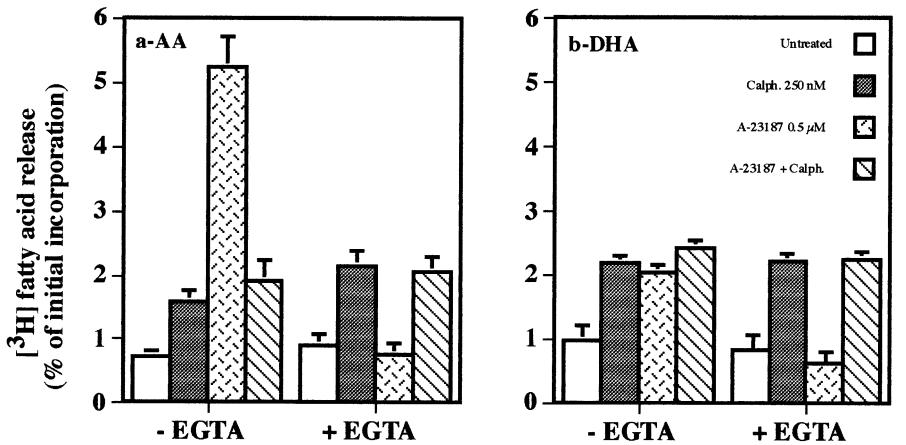

Fig. 4. Effect of extracellular Ca^{2+} depletion on basal, calphostin C-, A23187- and calphostin C/A23187-induced [^{3}H]AA or [^{3}H]DHA release from U_{III} cells. Cells labelled with [^{3}H]AA or [^{3}H]DHA were preincubated for 45 min with or without (untreated) 250 nM calphostin C, washed and then incubated with medium only or with 0.5 μM A23187 for 15 min, in the absence (−EGTA) or presence (+EGTA) of 3 mM EGTA. [^{3}H]AA (a) or [^{3}H]DHA (b) released in the medium was measured. Data are expressed as the percentage of the total radioactivity initially incorporated in cells, means ± S.D. of three independent measurements.

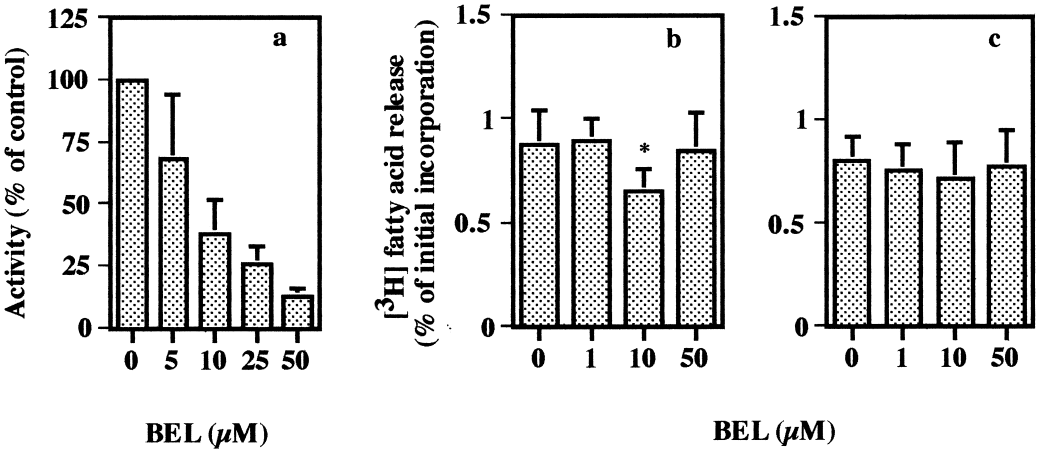

Fig. 5. Effect of BEL on the Ca^{2+}-independent PLA_2 activity (a) and on the basal release of [^{3}H]AA or [^{3}H]DHA (b, c). U_{III} cells were preincubated with the indicated concentrations of BEL for 30 min (a). Then enzyme activity was measured as described in Section 2 and expressed as the percent of control (enzyme activity in untreated cells, means ± S.D. of three independent measurements). Cells labelled with [^{3}H]AA or [^{3}H]DHA were preincubated for 30 min with BEL at various concentrations and washed before [^{3}H]AA (b) or [^{3}H]DHA (c). The release in the medium for 15 min was measured in the presence of 3 mM EGTA and 100 μM BAPTA. Data, expressed as the percentage of the total radioactivity initially incorporated in cells, are means ± S.D. of three independent measurements. *Significantly different ($P < 0.01$) from the control.

compared to merthiolate, suggests that acyltransferases are not involved in the stimulation of AA and DHA release by PKC inhibitors.

3.4. Ca^{2+}-independent PLA_2 activity in U_{III} cells

We next tested the hypothesis that a Ca^{2+}-independent phospholipase A_2 was involved. Well characterized intracellular Ca^{2+}-independent PLA_2 are limited to the 80-kDa Group VI $iPLA_2$ present in P388D1 macrophages [16] and CHO cells [17], a 40-kDa $iPLA_2$ present in myocardial tissue and pancreatic islets [15,34] and the 29-kDa Group VIII enzyme, which is a PAF acetylhydrolase [35]. One common feature of the 80- and the 40-kDa $iPLA_2$ is their selective and irreversible inhibition by the mechanism-based inhibitor BEL [15,36]. This compound exhibits an over 1000-fold selectivity for the $iPLA_2$ versus the Ca^{2+}-dependent PLA_2 [15] and does not affect a number of enzyme activities directly involved in AA metabolism such as arachidonoyl-CoA: lysophospholipid acyltransferase and CoA-independent transacylase [37]. The $iPLA_2$ activity from low speed supernatant (LSS) of U_{III} cell homogenate was assayed with 120 μM phospholipid substrate alone (vesicles) or in the presence of increasing amounts of Triton X-100 (mixed micelles). 1,2-dipalmitoyl-PC (DPPC) was used as substrate and the assays were carried out in the presence or absence of 0.8 mM ATP. A $iPLA_2$ activity was detected in the LSS. In the absence of added ATP, maximal activity was observed at a Triton X-100 concentration of 1.2 mM and was about 4-fold greater than that observed with vesicles. Addition of 0.8 mM ATP resulted in an additional increase of enzyme activity, about 3-fold in the absence of Triton X-100 and about 2-fold in the presence of 1.2 mM Triton X-100. Thus, maximal enzyme activity was observed with the combination of 1.2 mM Triton X-100 and 0.8 mM ATP and was roughly 9-fold greater than the activity observed with vesicles of DPPC (35.8 ± 5.2 pmol/min/mg protein and 4.3 ± 0.3 pmol/min/mg protein, respectively).

The involvement of a $iPLA_2$ in agonist-induced AA release has been suggested in different cells [38,39], based on BEL inhibition of AA release. In macrophages however, BEL has been shown to increase AA release [36]. The effect of BEL on $iPLA_2$ activity in U_{III} cells is shown in Fig. 5. Preincubation of cells for 30 min with increasing amounts of BEL resulted in a dose-dependent inhibition of enzyme activity with an apparent IC_{50} of about 7 μM (Fig. 5a). However, this inhibitory effect was not correlated with a dose-dependent inhibition of AA or DHA release (Fig. 5b and c). Only a small decrease of AA release was seen with 10 μM BEL, but a higher concentration produced no effect. The lack of a marked inhibitory effect of BEL on AA and DHA release indicates that the BEL-sensitive $iPLA_2$ does not significantly contribute to this release in U_{III} cells. When cells were preincubated with calphostin C no significant change in this enzyme activity was observed (not shown), in contrast with AA release. Moreover, when cells were preincubated with 50 μM BEL and 250 nM calphostin C, the enzyme activity was reduced by about 90% as in cells treated with BEL alone, but the basal release of AA was stimulated to the same extent as in cells treated with calphostin C alone (not shown).

The absence of correlation between $iPLA_2$ inhibition and fatty acid release together with the fact that the stimulation of basal AA release by calphostin C persists in cells pretreated with BEL suggest another releasing pathway. Another PLA_2, Ca^{2+}-independent but BEL-insensitive, might be involved. Horrocks and colleagues [40,41] have identified in bovine brain two $iPLA_2$ with molecular masses of 110 and 39 kDa that appeared to differ from myocardial and macrophage enzymes. The 39 kDa PLA_2 selectively acts on 1-alk-1′-enyl-2-acyl-sn-glycero-3-phosphoethanolamine (ethanolamine plasmalogens); it is strongly inhibited by glycosaminoglycans but is insensitive to BEL. A uterine enzyme with similar properties could be a good candidate since, in U_{III} cells, DHA is mainly incorporated in ethanolamine plasmalogens [20], and its release (Fig. 4b) is stimulated by the PKC inhibitor calphostin C in the absence of calcium.

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