Binding and internalization of extracellular type-I phospholipase A₂ in uterine stromal cells

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The cellular uptake of extracellular type-I phospholipase A₂ (PLA₂) was investigated in rat uterine stromal cells (U_{III}) in culture, which were found to express the high-affinity binding site for mammalian type-I PLA₂, with a measured K_D of 6.4 nM, a B_{max} of 0.1–1 pmol/mg of DNA at 4 °C, and a molecular mass of about 200 kDa. When U_{III} cells were treated with type-I PLA₂ at 37 °C, the ligand specifically associated with the cells increased, reaching a plateau after 90 min of incubation, whose level was about 5-fold higher than that measured if cells were maintained at 4 °C. We could determine that the PLA₂ was bound to plasma membrane receptors which were responsible for internalization of the ligand, and that the binding sites were still suitable for binding at the level of plasma membrane during U_{III} cell

incubation at 37 °C. Proteolysis of internalized PLA₂ could be clearly detected only after 90 min of U_{III} cell incubation with the ligand at 37 °C, and most of the intracellular PLA₂ consisted of the apparently intact 14 kDa enzyme. By cross-linking studies, we found that most of the internalized PLA₂ was not associated with the receptor, supporting the conclusion that in our experimental system a single pool of membrane receptors for mammalian type-I PLA₂ undergoes cycles of ligand binding, intracellular transfer and release of PLA₂, followed by restoration of binding sites on the plasma membrane. We calculated that the rate of internalization of the ligand by one receptor molecule in U_{III} cells at 37 °C is about three molecules of type-I PLA₂ per h.

INTRODUCTION

Phospholipases A₂ (PLA₂s; EC 3.1.1.4) are lipolytic enzymes that hydrolyse the sn-2-acyl ester bond in glycerophospholipid molecules, releasing a free fatty acid and a lysophospholipid [1]. PLA₂ plays a central role in a variety of cellular processes, including phospholipid metabolism and signal transduction [2–4]. In the latter instance, the liberation of arachidonic acid by PLA₂ is of paramount importance, as this fatty acid is the substrate of cyclo- and lipoxygenases, generating the various eicosanoids [5,6]. Furthermore, the lysophospholipids released by PLA₂ can represent important signalling molecules, as they may be precursors of several lipid mediators [5–9].

Many forms of PLA₂ enzyme have been described, and are classified into several groups [1,3,4]. Type-I, -II and -III PLA₂s comprise extracellular, low-molecular-mass (13–18 kDa) enzymes, including secretory pancreatic and cobra venom PLA₂s (type I), rattlesnake and synovial fluid PLA₂s (type II), and bee venom PLA₂ (type III). Intracellular, cytosolic PLA₂s, instead, belong to different groups, including the 85 kDa (type IV) and 40 kDa enzymes [3,4].

In recent years, evidence on the involvement of distinct types of PLA₂ in inflammatory responses [3,6,10–12] and cell proliferation [13–16] has been obtained, but the functional roles specifically played by the different PLA₂ forms have not been clarified yet. Furthermore, the molecular mechanisms involved in the control of PLA₂ functioning in mammalian systems are poorly understood. For instance, while activation of cytosolic 85 kDa PLA₂ and liberation of arachidonic acid have been linked to agonist-induced responses in several cell lines [16–18], it has been also shown that type-I PLA₂ is able to stimulate cell proliferation by binding to a membrane receptor [13,14].

The role of PLA₂ in the control of cell functions attracted our attention after we found that the proliferation of a uterine stromal cell line established in our laboratory (U_{III} cells) requires

an intact PLA₂ pathway, but is not affected by inhibitors of cyclo- and lipoxygenase [19], suggesting that either the fatty acids or the lysophospholipids liberated by PLA₂ were involved in the phenomenon. Subsequently, we obtained evidence that U_{III} cell proliferation is affected by unsaturated fatty acids, with arachidonic acid being a stimulator of the process [20]. We then decided to expand the investigation on to the functioning of PLA₂ in uterine stromal cells by evaluating the interaction of type-I PLA₂ with our experimental system.

In this paper we show that uterine stromal cells express membrane receptors for mammalian type-I PLA₂ and internalize the enzyme bound to receptors.

EXPERIMENTAL

Materials

Na¹²⁵I (carrier-free, 100 mCi/ml) was purchased from DuPont–New England Nuclear. Dithiobis(succinimidylpropionate) (DSP) and PLA₂s from porcine and bovine pancreas, and from *Crotalus atrox*, *Naja mocambique mocambique* and bee venoms were obtained from Sigma. Disuccinimidylsuberate (DSS) and Iodo-Beads were purchased from Pierce Chemical Co. The media and reagents for cell culture were obtained from TechGen International (Les Ulis, France). Prestained molecular-mass markers (Kaleidoscope prestained standards) used in SDS/PAGE have been purchased from Bio-Rad. All other reagents were of analytical grade.

Iodination of PLA₂

Iodination of porcine pancreatic PLA₂ and *Crotalus atrox* venom PLA₂ was carried out using the Iodo-Beads iodinating reagent, according to the Manufacturer's instructions. The reaction was terminated by removal of the PLA₂ solution from the reaction vessel, and the separation of iodinated PLA₂ from unincor-

Abbreviations used: PLA₂, phospholipase A₂; DSS, disuccinimidylsuberate; DSP, dithiobis(succinimidylpropionate); TCA, trichloroacetic acid.

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porated ^{125}I was performed by gel-filtration, using a PD-10 column (Pharmacia) equilibrated and eluted with 20 mM phosphate buffer, pH 7.4, 0.15 M NaCl (PBS). The specific radioactivity of iodinated PLA_2 was 100–200 d.p.m./fmol.

Cell culture

Rat uterine stromal cells (U_{III} cells) were grown in 60-mm-diam. Petri dishes in 5% CO_2 in air at 37 °C. The culture medium consisted of M199 medium containing 10% (v/v) fetal-calf serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, as described previously [21].

Binding studies

Confluent cells were used throughout the entire study. The cells in Petri dishes were washed once with 2.5 ml of binding medium, consisting of M199 medium containing 0.1% (w/v) BSA and 50 mM Hepes buffer, pH 7.2. Culture dishes received 1.5 ml of binding medium, and cells were then incubated with the indicated concentrations of iodinated PLA_2 and in the presence or in the absence of a 100-fold molar excess of non-radioactive PLA_2 as competitor. If not stated otherwise, the incubation with ligand was performed for 2 h at 4 °C. At the end of the incubation, the binding medium was removed, and cells were washed five times with 2.5 ml of PBS buffer at 4 °C. The radioactivity associated with U_{III} cells was then determined after solubilization with 1 ml of 1 M NaOH and sample neutralization with 1 ml of 1 M HCl. The specific binding of PLA_2 to U_{III} cells was determined by subtraction of the radioactivity detected in the samples from cells incubated in the presence of competitor from that of paired samples incubated in its absence. Analysis of specific binding data was performed according to Scatchard [22].

Cross-linking of intact cells

U_{III} cells were incubated with ligand as specified in the text, were washed twice with PBS, and were harvested by scraping. Cells were then recovered by low-speed centrifugation, and were dispersed with 1 ml of PBS. Cross-linking was performed by incubation of cells suspensions with the indicated reagent for 60 min at 2 °C, and blocking the reaction by the addition of lysine at a final concentration of 100 mM and incubation for 30 min at 2 °C, as previously described [23]. The cross-linkers we have used were either DSS, or DSP, or glutaraldehyde, at a final concentration of 1 mM, 2 mg/ml and 0.025% (w/v) respectively. After cross-linking, cells were recovered, washed once with PBS and were processed to prepare total soluble extracts.

Preparation of total soluble extracts from U_{III} cells

Washed cells were homogenized by suspension with 0.4 ml of 20 mM Tris/HCl, pH 7.5 at 2 °C/2 mM EDTA/1% (w/v) Triton X-100, and sonicated twice for 10 s at 2 °C with a Branson B-15 sonifier with an output of 15 W. Aliquots (20 μl) of the homogenate were saved and used for the determination of DNA, according to the method of Labarca and Paigen [24]. The homogenate was centrifuged for 1 h at 105000 g, and the supernatant of this centrifugation represented the total soluble extract used in further analyses.

Analysis of cell extracts by SDS/PAGE

Total soluble extracts from U_{III} cells were brought to a final 2% (w/v) SDS concentration, and were treated for 10 min at 100 °C, before being fractionated by SDS/PAGE according to the method of Laemmli [25], and using 5–20% gradient gels. Proteins were electrophoretically transferred to nitrocellulose Protran BA

83 membranes (Schleicher & Schuell), and autoradiography of ^{125}I -labelled material adsorbed on to the membrane was performed using Hyperfilm- ^3H (Amersham).

RESULTS

Analysis of binding of type-I PLA_2 to uterine stromal cells

When confluent U_{III} cells were incubated with increasing concentrations of type-I PLA_2 at 4 °C (Figure 1), the specific binding measured reached a plateau at concentrations higher than 10 nM. Scatchard transformation of binding data revealed the presence of a single class of binding sites with a measured K_D of $(6.4 \pm 2.8) \times 10^{-9}$ M ($n = 4$) and a B_{max} ranging from 0.1 to 1 pmol/mg of DNA.

In order to evaluate the specificity of these binding sites, U_{III} cells were incubated with iodinated porcine pancreatic PLA_2 and in the presence of non-radioactive competitor PLA_2 s obtained from different sources. The results we obtained (Figure 2) showed that this class of binding sites preferentially bound mammalian type-I PLA_2 , inasmuch as cobra venom (type I), type-II (from *Crotalus atrox* venom) or type-III (from bee venom) PLA_2 s were

Figure 1 Analysis of type-I PLA_2 binding in U_{III} cells

(A) Cells were incubated for 2 h at 4 °C with increasing concentrations of iodinated porcine pancreatic PLA_2 and in the presence, or in the absence, of a 100-fold molar excess of non-radioactive PLA_2 . At the end of the incubation, cells were washed, and the radioactive PLA_2 associated with U_{III} cells was determined. The specific PLA_2 binding ($\blacktriangle$) was calculated by subtraction of binding obtained in the presence of competitor ($\circ$) from that found in its absence ($\bullet$). (B) Scatchard plot of specific PLA_2 binding.

Figure 2 Specificity of PLA₂ binding in U_{III} cells

Cells were incubated for 2 h at 4 °C with 20 nM iodinated porcine pancreatic PLA₂ and either in the absence, or in the presence, of the indicated concentrations of the following non-radioactive competitors: type-I PLA₂ obtained from either porcine (○) or bovine (●) pancreas, and from the venom of *Naja mocambique mocambique* (▲); type-II PLA₂ obtained from *Crotalus atrox* venom (◆); and type-III PLA₂ obtained from bee venom (■). The specific binding was determined as described in the legend to Figure 1, and was expressed as the percentage of that found by subtraction of binding measured in the presence of 2 μM non-radioactive porcine pancreatic PLA₂ from that detected in the absence of competitor. The binding data obtained with non-radioactive PLA₂ from *Naja mocambique mocambique*, *Crotalus atrox*, and bee venoms, represent means ± S.E.M. of values determined in four separate experiments.

weaker competitors than pancreatic type-I PLA₂ for binding to uterine cells.

Analysis of PLA₂ binding sites by chemical cross-linking

In order to further characterize these binding sites, U_{III} cells were incubated with iodinated pancreatic PLA₂, and were then treated, or not, with the cross-linker DSS, to induce the formation of covalent bonds between PLA₂ and its receptor. As shown in Figure 3, SDS/PAGE analysis of cell extracts revealed that a radioactive band with an electrophoretic mobility corresponding to a molecular mass of 210 ± 6 kDa ($n = 5$) could be detected in samples from DSS-treated cells but not from control cells. As the detection of this entity was abolished when extracts were prepared from cells which were incubated with iodinated PLA₂ in the presence of non-radioactive competitor, our data show that the 210 kDa component indeed represents the PLA₂-receptor complexes formed in intact U_{III} cells. The detection of this 210 kDa species did not depend on the non-reducing conditions we employed in SDS/PAGE, as a band displaying the same electrophoretic mobility was found when the analysis was carried out after sample treatment with 5% 2-mercaptoethanol (results not shown). We also observed that the detection of a broad band of free PLA₂ (Figure 3) was not affected by 2-mercaptoethanol treatment of samples. Indeed, in some experiments two different molecular forms of PLA₂ of 20 and 14 kDa could be resolved (see, for instance, Figure 9), confirming the heterogeneity of pancreatic PLA₂ detectable by SDS/PAGE [26,27]. Furthermore, the experiments we have performed so far, have shown that both

Figure 3 Characterization of PLA₂ binding sites by cross-linking of U_{III} cells

Cells were incubated for 2 h at 4 °C with 20 nM iodinated porcine pancreatic PLA₂ and in the presence (lanes 2 and 4), or in the absence (lanes 1 and 3), of a 100-fold molar excess of non-radioactive competitor. At the end of the incubation, cells were washed and were subjected (lanes 3 and 4), or not (lanes 1 and 2), to cross-linking with DSS, as described under the Experimental section. Cells were then recovered, homogenized, and total soluble extracts were prepared and subjected to SDS/PAGE analysis. The prestained molecular mass markers were: myosin (202 kDa), β-galactosidase (134 kDa), BSA (71 kDa), carbonic anhydrase (41.8 kDa), and soybean trypsin inhibitor (30.6 kDa).

forms can be specifically associated with binding sites, and that their levels reflect their relative proportions in different batches of ligand, rather than a discrimination of molecular forms by the binding site.

Internalization of type-I PLA₂ in U_{III} cells

In order to gain insights into the functioning of PLA₂ receptors under physiological conditions, we first allowed formation of PLA₂-receptor complexes at low temperature, and then continued the incubation at either 4 or 37 °C. As it is shown in

Figure 4 Time-course of PLA₂ association with U_{III} cells

Cells were preincubated for 2 h at 4 °C with 20 nM iodinated porcine pancreatic PLA₂ in the presence, or in the absence, of a 100-fold molar excess of non-radioactive competitor, and the incubation was then continued at either 4 (○) or 37 °C (●). At the indicated times, cells were processed to determine the levels of radioactive PLA₂ specifically associated with U_{III} cells, as described in the legend to Figure 1.

Figure 5 Time-course of PLA₂ association with the plasma membrane and the intracellular compartment of U_{III} cells at 37 °C

Cells were preincubated for 2 h at 4 °C with 20 nM iodinated porcine pancreatic PLA₂ in the presence, or in the absence, of a 100-fold molar excess of non-radioactive competitor, and the incubation was then continued at 37 °C. At the indicated times, cells were washed, and were incubated with 50 mM glycine buffer, pH 3.0, containing 0.1 M NaCl, for 10 min at 4 °C. The acidic buffer was then collected and used to determine the acid-extractable, membrane-bound radioactivity (●). Cells were then solubilized with 1 M NaOH, to measure the residual acid-resistant, intracellular radioactivity (▲), as described in the Experimental section. The levels of PLA₂ specifically associated with the two fractions were determined, as described in the legend to Figure 1. The sum of acid-extractable and acid-resistant fractions represented the total (■) levels of PLA₂ specifically associated with U_{III} cells.

Figure 4, the temperature shift to 37 °C induced a progressive increase in the levels of PLA₂ specifically associated with U_{III} cells, reaching a plateau after 90 min, which was maintained after 3 h of incubation at 37 °C (results not shown). This increase was not due to a lack of attainment of the equilibrium of the binding reaction at low temperature, as incubation of paired samples at 4 °C showed that steady-state levels of PLA₂ binding had been already reached before the temperature shift to 37 °C (Figure 4).

The temperature-induced increase in PLA₂ associated with U_{III} cells led us to evaluate the possibility that it might have been due to internalization and recycling of receptors and/or appearance of new binding sites on the plasma membrane. We then took advantage of the fact that cell washing with acidic buffers remove the PLA₂ bound to plasma membrane receptors [28], and measured the time-course of PLA₂ levels detectable in the extracellular (acid-soluble) and intracellular (acid-resistant) compartments of U_{III} cells. The results reported in Figure 5 have been obtained in a typical experiment, and show that most radioactivity associated with U_{III} cells is located on the extracellular side of the plasma membrane at the end of preincubation with iodinated type-I PLA₂ at 4 °C, and that this fraction remained essentially constant over the time-course of incubation at 37 °C. Indeed, the levels of PLA₂ associated with plasma membrane receptors after 90 min of incubation at 37 °C were $110 \pm 20\%$ ($n = 7$) of those measured at the beginning of the incubation. The intracellular PLA₂, instead, progressively increased, reaching a maximum within 90 min of incubation at 37 °C, whose levels

Figure 6 Pulse-chase of PLA₂ association with the plasma membrane and the intracellular compartment of U_{III} cells

Cells were preincubated for 2 h at 4 °C with 20 nM iodinated porcine pancreatic PLA₂ in the presence, or in the absence, of a 100-fold molar excess of non-radioactive competitor. (A) At the end of the preincubation, cells were subdivided into two sets. Non-radioactive PLA₂ was added to cell samples of the first set (open symbols) at a 2 μM final concentration, whereas cell samples of the second set (closed symbols) received no addition. The incubation was then continued at 37 °C, and at the indicated times cells were processed to determine the levels of PLA₂ specifically associated with plasma membrane (○, ●) and the intracellular compartment (△, ▲) of U_{III} cells, as described in the legend to Figure 5. (B) At the end of the preincubation, cells were subdivided into two sets. The cells of the first set (closed symbols) received no addition and the incubation was continued at 37 °C. At the indicated times, cells were processed to determine the levels of PLA₂ specifically associated with plasma membrane (●) and the intracellular compartment (▲), as described above. The cells of the second set (open symbols) were further incubated for 90 min at 37 °C, receiving a 2 μM final concentration of non-radioactive PLA₂ at either the beginning of the incubation or 20, 40 and 60 min after the beginning of the incubation at 37 °C. At the end of the incubation, the levels of PLA₂ specifically associated with plasma membrane (○) and the intracellular compartment (△) of U_{III} cells was determined, as described above.

were about 4-fold (3.9 ± 0.5 ; $n = 7$) higher than those of its extracellular counterpart (Figure 5). These findings implied that PLA₂ was internalized in U_{III} cells at physiological temperature, and that PLA₂ receptors were maintained available for binding at the level of plasma membrane. The presence of 1 mM

Figure 7 Time-course of PLA₂ internalization (A) and degradation (B) in U_{III} cells at 37 °C

Cells were preincubated for 2 h at 4 °C with 20 nM iodinated porcine pancreatic PLA₂ in the presence, or in the absence, of a 100-fold molar excess of non-radioactive competitor. At the end of the preincubation, cells were washed five times with PBS, and were then incubated with 1.5 ml of binding medium at 37 °C. (A) At the indicated times, the medium was removed and used for determination of the radioactivity released from U_{III} cells (○). Cells were then processed to measure the levels of PLA₂ specifically associated with the plasma membrane (●) and the intracellular compartment (▲), as described in the legend to Figure 5. (B) The medium recovered from dishes was brought to a final 10% (w/v) TCA concentration, and the TCA-precipitable (●), and TCA-soluble (○) radioactivity was determined.

cycloheximide during incubation of U_{III} cells at 37 °C, a condition which blocks protein synthesis in uterine cells [29], did not affect the levels of PLA₂ specifically associated with both the plasma membrane and intracellular compartment (results not shown), implying that synthesis of new receptor proteins was not involved in the phenomenon we have observed.

In order to evaluate further the internalization of PLA₂ in U_{III} cells, we carried out pulse-chase experiments (Figure 6). When a 100-fold excess of non-radioactive PLA₂ was added to our samples at the end of the preincubation with iodinated PLA₂ at

Figure 8 Time-course of the association of type-I and type-II PLA₂ with U_{III} cells at 37 °C

Cells were preincubated for 2 h at 4 °C with 20 nM of iodinated ligand, consisting of either porcine pancreatic (type-I) PLA₂ (closed symbols), or type-II PLA₂ from *Crotalus atrox* venom (open symbols), in the presence or in the absence of a 100-fold molar excess of the respective non-radioactive competitor, and the incubation was continued at 37 °C. At the indicated times, cells were processed to determine the levels of PLA₂ specifically associated with the plasma membrane (○, ●) and the intracellular compartment (△, ▲) of U_{III} cells, as described in the legend to Figure 5.

4 °C, and the incubation was continued at 37 °C, the levels of radioactive PLA₂ bound to membrane receptors progressively declined, with a $t_{1/2}$ of about 20 min, and the increase of the intracellular iodinated PLA₂ was prevented (Figure 6A). Interestingly, the addition of non-radioactive PLA₂ to U_{III} cells did not result in a progressive decline of intracellular PLA₂, whose levels measured at the end of the chase periods essentially coincided with those found at the beginning of the chase (Figure 6B).

These results suggested that most PLA₂ internalized in U_{III} cells was not degraded and released in the incubation medium over the time-course of our experiments. We then directly evaluated this issue by allowing formation of PLA₂-receptor complexes at 4 °C, followed by removal of unbound ligand and by measurements of the distribution of radioactive PLA₂ upon cell incubation at 37 °C. The results shown in Figure 7(A) show that, in the absence of an exogenous supply of PLA₂, the membrane-bound species rapidly decline, paralleled by an increase of radioactive PLA₂ in the incubation medium and inside U_{III} cells. The intracellular content of PLA₂ reached a plateau within 20 min, and remained constant over the next 70 min of incubation at 37 °C, confirming that stable levels of intracellular PLA₂ are maintained by U_{III} cells under our experimental conditions (Figure 6). The increase in the levels of radioactivity in the incubation medium, instead, was found to follow a biphasic pattern (Figure 7A), including a rapid accumulation in the first 30 min, followed by a modest increment in the next 60 min of incubation at 37 °C. Aliquots of incubation medium were then treated with trichloroacetic acid (TCA), to distinguish between intact (TCA-precipitable) and degraded (TCA-soluble) PLA₂. As shown in Figure 7(B), we found that the first phase of increase in radioactivity in the medium was due to release of

Figure 9 Time-course of PLA₂ bound to receptor in U_{III} cells at 37 °C

Upper panel: cells were preincubated for 2 h at 4 °C with 20 nM iodinated porcine pancreatic PLA₂ in the presence (lanes 2 and 8), or in the absence (lanes 1, 3–7), of a 100-fold molar excess of non-radioactive competitor, and the incubation was then continued at 37 °C. After 0 (lanes 1 and 2), 15 (lane 3), 30 (lane 4), 45 (lane 5), 60 (lane 6), and 90 min (lanes 7 and 8) of incubation at 37 °C, cells were washed and were then subjected to cross-linking with DSS. After cross-linking, cells were processed to prepare total soluble extracts, which were analysed by SDS/PAGE. The molecular-mass standards were as described in the legend to Figure 3. Lower panel: the portions of the membrane used for the autoradiography shown in the upper panel, corresponding to the 210 kDa (○) and 14 kDa (●) components, were excised and were used for determination of radioactivity, which has been expressed as the n-fold increase, compared with that found for each component at the beginning of the incubation at 37 °C.

intact PLA₂, reaching levels which remained constant in the following incubation period, when low levels of degraded PLA₂ could be detected.

Taken together, these results indicated that PLA₂ was internalized by its own receptor in U_{III} cells. In order to ascertain that intracellular accumulation of type-I PLA₂ actually involved the receptor and was not due to diffusion of the free enzyme through plasma membrane, we took advantage of the fact that the receptor showed a very low affinity for type-II PLA₂ (Figure 2), whose molecular mass and chemical properties are very similar to those of type-I PLA₂ [30,31]. U_{III} cells were then incubated with either type-I or type-II PLA₂ for different times at 37 °C, and the levels of plasma membrane and intracellular enzyme

were determined. The results we obtained (Figure 8), confirmed the distribution and intracellular accumulation of type-I PLA₂ we had already observed (Figures 5–7), and showed that, under the same conditions, background quantities of type-II PLA₂ remained associated with U_{III} cells, independently of the time of incubation at 37 °C. Furthermore, we also observed that a 100-fold molar excess of non-radioactive type-II PLA₂ could not prevent internalization of type-I PLA₂ (results not shown). We could then conclude that internalization of pancreatic, type-I PLA₂ in U_{III} cells occurs via its own membrane receptor.

Most PLA₂ internalized in U_{III} cells is detected as free ligand

In order to gain further insights into the mechanism of PLA₂ accumulation inside U_{III} cells, we undertook experiments to evaluate whether membrane receptors were internalized with the ligand. This aspect was analysed by cross-linking of U_{III} cells, taking advantage of the fact that DSS can diffuse through the plasma membrane [32], and should then stabilize intracellular PLA₂–receptor complexes. To this end, we incubated U_{III} cells with iodinated PLA₂ for different times at 37 °C, cells were then subjected to cross-linking with DSS, and total cellular extracts were prepared. The autoradiography we obtained after these extracts had been analysed by SDS/PAGE (Figure 9, upper panel), revealed that the levels of PLA₂–receptor complexes stabilized by cross-linking did not greatly change over the time of U_{III} cell incubation at 37 °C, whereas a net increase of free PLA₂ was clearly detectable. Interestingly, this increase involved intact 14 kDa PLA₂, confirming that degradation of the enzyme was limited under our experimental conditions. When the bands corresponding to the free and receptor-bound forms of the enzyme were excised and counted (Figure 9, lower panel), the data we obtained showed that the time courses of the two forms of PLA₂ are similar to that observed for the intracellular and membrane-associated pools respectively (Figures 5–8).

While the results shown in Figure 9 indicated that most PLA₂ associated with U_{III} cells at 37 °C is not bound to its receptor, they did not allow a direct distinction of intracellular and extracellular (membrane-bound) fractions. In order to distinguish the two pools, we performed cross-linking of U_{III} cells after the PLA₂ bound extracellularly had been released, or not, by the acidic wash.

As shown in Figure 10, the acidic wash of cells exposed to PLA₂ at 4 °C resulted in disappearance of the 210 kDa PLA₂–receptor complexes stabilized by cross-linking, and in detection of background levels of the free enzyme (compare lanes 1 and 5), confirming that under these conditions PLA₂ is essentially extracellular, bound to membrane receptors. When U_{III} cells incubated with PLA₂ at 37 °C were subjected to the acidic wash, instead, high levels of free PLA₂ were still observed, whereas 210 kDa PLA₂–receptor complexes were barely detectable (compare lanes 3 and 7). These findings would indicate that only a small fraction of receptor-bound PLA₂ in U_{III} cells incubated at 37 °C is resistant to the acidic wash and is then intracellular, but may also simply reflect the inefficacy of DSS in stabilizing intracellular PLA₂–receptor complexes. In order to check this latter possibility, we repeated the experiment using two different cross-linkers, glutaraldehyde and DSP, under conditions which have been shown to efficiently stabilize intracellular protein–protein interactions [23,33–35]. The results we obtained by treatment of intact U_{III} cells with these two cross-linkers were identical to those observed with DSS (results not shown), confirming the conclusion that very low levels of PLA₂–receptor complexes can be detected inside U_{III} cells incubated with ligand at 37 °C.

Figure 10 Analysis of PLA₂-receptor complexes prepared from acid-treated and untreated U_{III} cells

Cells were preincubated for 2 h at 4 °C with 20 nM iodinated porcine pancreatic PLA₂ in the presence (lanes 2,4,6 and 8), or in the absence (lanes 1,3,5 and 7), of a 100-fold molar excess of non-radioactive competitor, and the incubation was then continued for 90 min at either 4 (lanes 1,2,5 and 6) or 37 °C (lanes 3,4,7 and 8). At the end of the incubation, cells were subjected (lanes 5–8), or not (lanes 1–4), to the acidic wash as described in the legend to Figure 5, were washed with PBS, and were subjected to cross-linking with DSS. After cross-linking, cells were processed to prepare total soluble extracts, which were analysed by SDS/PAGE. The molecular-mass markers were as described in the legend to Figure 3.

DISCUSSION

Several mammalian cells and tissues express receptors for secretory-type, extracellular PLA₂s, whose K_D values measured with type-I pancreatic PLA₂ vary in the 10⁻⁹–10⁻⁸ M range [13,14,28,36–39]. The receptor we have detected in a rat uterine stromal cell line (U_{III}) displays many characteristics of these receptors, such as a K_D for pancreatic PLA₂ of about 6 nM (Figure 1), a lower affinity for snake and bee venom PLA₂s (Figure 2), and a molecular mass of about 200 kDa when it is complexed with the ligand (Figure 3). According to these findings, we can conclude that U_{III} cells express the membrane receptor for extracellular type-I PLA₂.

The $B_{\max}$ we measured in U_{III} cells at 4 °C, corresponding to 2–20 fmol/10⁶ cells, or 1500–15000 sites/cell, is also in the range of those found in other rat tissues and cells [28]. Interestingly, the 10-fold variation observed in the experiments we have performed, was found to depend on the cell density and proliferative state of U_{III} cells (J.-M. Fayard, G. P. Rossini, C. Tessier and C. Laugier, unpublished work). This type of observation is in line with the changes in receptor levels detected with rabbit skeletal muscle cells during their maturation process [36], and with the detection of heterogeneous and homologous regulation of PLA₂ binding in vascular smooth-muscle cells [28], suggesting that the level of PLA₂ receptors expressed in different cells is physiologically relevant.

The early phases of PLA₂ receptor functioning in intact U_{III} cells have thus attracted our attention, and we found that the ligand was accumulated and internalized at 37 °C (Figures 4–8). The involvement of PLA₂ receptors in the phenomenon is supported by several criteria, as: (i) the determination of PLA₂ levels in U_{III} cell fractions was always performed by subtraction of radioactivity detected in the presence of non-radioactive competitor from that found in its absence, and (ii) PLA₂ from *Crotalus atrox* venom, which displays characteristics very similar to those of pancreatic PLA₂ [30,31] but does not efficiently bind to receptors of U_{III} cells (Figure 2), is not internalized at a

significant extent under our experimental conditions (Figure 8) and cannot prevent the internalization of pancreatic PLA₂.

Most of the ligand internalized in our experimental system is not extensively degraded in the first 60–90 min of incubation at 37 °C (Figure 7), so that high levels of the apparently intact protein can be found in the intracellular compartment (Figure 10). A time-lag of 30 and 90 min before detection of proteolytic fragments of PLA₂ in the incubation medium has been also observed in other experimental systems [28,39], and differences most likely reflect cell-specific patterns of controlled PLA₂ degradation.

The molecular mechanisms of receptor-mediated internalization of extracellular PLA₂ have not been extensively investigated in previous studies. The results we have obtained showed that intracellular accumulation of PLA₂ was not accompanied by depletion of membrane receptors (Figure 5). As inhibition of protein synthesis by cycloheximide did not affect the phenomenon, the maintenance of the plasma membrane receptor pool should represent recovery of binding sites after internalization of bound PLA₂. The molecular mechanisms of this process should then involve either recruitment of new binding sites from an inactive (or an intracellular) pool, substituting for the receptor molecules internalized with the ligand, or rapid recycling of a single pool of membrane receptors. In this latter case, the phenomenon would involve PLA₂ binding to membrane receptors, intracellular transfer and release of bound ligand, and restoration of plasma membrane binding sites. The two mechanisms could be experimentally distinguished in cross-linking studies, as the former should involve detection of increased levels of PLA₂-receptor complexes and recovery of significant amounts of intracellular receptor upon internalization of the ligand, whereas the latter would result in constant levels of PLA₂-receptor complexes without their significant recovery in the intracellular compartment during ligand internalization.

The results we obtained by cross-linking of U_{III} cells, revealed that internalization of PLA₂ is not accompanied by an increase in PLA₂-receptor complexes (Figure 9), and that very low levels of receptor complexes are detectable in the intracellular compartment of uterine cells (Figure 10).

Several lines of evidence would support the conclusion that these findings reflect the characteristics of the phenomenon occurring in intact U_{III} cells, rather than the inefficient cross-linking of intracellular PLA₂-receptor complexes by DSS. It is known that the efficiency of cross-linking is directly related to the concentration of the reagent and inversely related to that of the groups reacted upon by the cross-linker [40,41]. The possibility that our data might be due to different efficiencies of cross-linking among samples, however, can be excluded, as the paired samples subjected to our procedure contained the same quantity of cells and concentration of reagent, in the experiments we have performed. Two other possibilities are that DSS might not be appropriate to cross-link intracellular PLA₂-receptor complexes because of a lack of diffusion in adequate concentrations into U_{III} cells, or due to its chemical structure. Both possibilities, however, could be also excluded, as we could not detect significant levels of intracellular PLA₂-receptor complexes when cross-linking of U_{III} cells treated with iodinated PLA₂ at 37 °C was repeated using two different reagents, glutaraldehyde and DSP, under conditions which have been shown to efficiently cross-link intracellular components in several cell lines [23,33–35,41,42].

On the basis of these findings, the results we obtained support the conclusion that a single pool of plasma membrane receptors for type-I PLA₂ goes through cycles of ligand binding and its internalization in U_{III} cells. According to this interpretation, the rate of internalization of the ligand by one receptor molecule in