Cytosolic phospholipase A_2–p11 interaction controls arachidonic acid release as a function of epithelial cell confluence

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Madin–Darby canine kidney type II cells were shown to release low amounts of AA (arachidonic acid) and prostaglandin E_2 in response to various stimuli when analysed after cell confluence. In contrast, non-confluent Madin–Darby canine kidney type II cells released much higher amounts of AA and prostaglandin E_2. In both stationary and non-confluent cells, AA was released by type IV $cPLA_2$ (cytosolic phospholipase A_2), as shown by the use of specific inhibitors and by analysis of the profile of fatty acids released. This confluence-dependent $cPLA_2$ activation was not due to a difference in expression, or in phosphorylation of the enzyme, or in the amount of its substrate. To find out the mechanism by which $cPLA_2$ activation may be regulated as a function of cell confluence, immunofluorescence and co-immunoprecipitation experiments were performed using $cPLA_2$, p11, a natural inhibitor of the enzyme, and annexin II, the natural ligand of p11. These three proteins were expressed at a constant level, regardless of the cell confluence. In contrast, whereas annexin II and $cPLA_2$ interacted at a constant rate, p11 and $cPLA_2$ interacted more strongly in stationary cells, thus indicating that $cPLA_2$ activation is regulated by its accessibility to p11, independent of their expression level. Our results indicate that, in epithelial cells, the cell confluence, i.e. the establishment of cell–cell contacts, rather than cell proliferation directly controls $cPLA_2$ activation by changing the stoichiometry of p11/$cPLA_2$ interaction.

Key words: activation, annexin II, confluence, epithelial cell, p11, phospholipase A_2 (PLA_2).

INTRODUCTION

Establishment of cell–cell junctions is a key feature of epithelial cells that generate a selective barrier allowing the expression of epithelial-specific functions. In several pathological processes such as oncogenesis, normal contacts between epithelial cells are disrupted, and this loss of functional cell–cell junctions is followed by an enhanced cell proliferation. This is mainly due to the activation of several transduction pathways, leading to a transformed phenotype of epithelial cells. Among the changes observed, an increased eicosanoid production is described in numerous cancers [1], but the relationship between proliferation and this overproduction still remains unclear [2]. Several studies reported an increased eicosanoid production in actively proliferating cells as well as in cancer tissue [3] compared with stationary cells [4–6]. In the meantime, the mechanisms involved in this overproduction are not clear, although several groups, including ours, demonstrated an up-regulation of the enzymes involved in eicosanoid synthesis: cyclo-oxygenase-2, $sPLA_2$ (secretory Ca^{2+}-dependent phospholipase A_2) and $cPLA_2$ (cytosolic PLA_2), in human colon cancer tissue [7–10].

AA (arachidonic acid) release is the first rate-limiting step in the synthesis of eicosanoids. The major pathway for free AA production is the deacylation of membrane phospholipids in the *sn*-2 position by PLA_2. PLA_2s belong to a wide family of enzymes [11], including at least three groups in mammalian cells: $sPLA_2$s, $iPLA_2$s (intracellular Ca^{2+}-independent PLA_2s) and the 85 kDa Ca^{2+}-dependent $cPLA_2$. $sPLA_2$s and $iPLA_2$s are mainly involved in long-term production of eicosanoids and in membrane phospholipid remodelling respectively [12,13]. In some cases, these two enzymes are also capable of producing free AA, in activated cells for $sPLA_2$ [14] and after activation by caspase proteolysis for $iPLA_2$ [15]. However, numerous evidences indicate that $cPLA_2$ plays the main role in the rapid overproduction of lipid mediators in response to stimuli [16,17]. Activation of $cPLA_2$ was first shown to be dependent on at least two mechanisms: the phosphorylation of the enzyme by various kinases [18] and its translocation to membrane phospholipids by a Ca^{2+}-dependent lipid-binding domain, following an increase in intracellular Ca^{2+} (see [19] for a review). These two complementary mechanisms give this cytosolic enzyme access to its membrane substrate. However, $cPLA_2$ is tightly regulated by other mechanisms, including regulation of its expression at the transcriptional level, interaction with membrane lipids such as cholesterol [20], phosphatidylinositol bisphosphate [21] or ceramides [20, 22] and also through interaction with other proteins such as annexins [23,24], p11 (S100A10 calcium-binding protein) [25] or vimentin, an intermediate-filament protein [26]. Interestingly, several studies have also reported a PLA_2-dependent down-regulation of AA release in various cultured cells when they stop growing [4–6], suggesting that AA and its metabolites may be involved in the regulation of proliferation or apoptosis (see [27] for a review).

It is well known that epithelial cells in culture, in contrast with other cell types, are able to differentiate and to grow after confluence to establish a continuous monolayer of polarized cells

Abbreviations used: AA, arachidonic acid; $AACOCF_3$, arachidonyltrifluoromethyl ketone; Ab, antibody; AxII, annexin II; BEL (=HELSS), bromoenol lactone suicide substrate; Caco-2 cells, colon carcinoma-2 cells; FCS, foetal calf serum; mAb, monoclonal Ab; MDCK, Madin–Darby canine kidney; NEFA, non-esterified ('free') fatty acid; pAb, polyclonal Ab; PLA_2, phospholipase A_2; $cPLA_2$, $iPLA_2$, $sPLA_2$, cytosolic, intracellular Ca^{2+}-independent, secretory Ca^{2+}-dependent PLA_2 respectively.

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expressing specialized functions, reproducing the major features of epithelia *in situ*. In the present study, we used MDCK (Madin–Darby canine kidney) type II cells as a model of epithelial cells to study $cPLA_2$ regulation as a function of proliferation and cell–cell interaction. Our results show that AA release is down-regulated when cells reach confluence and that this is not related to the proliferation rate, but rather to the establishment of cell–cell contacts. In these epithelial cells, AA is mainly released by $cPLA_2$, but the down-regulation occurs under conditions where none of the following, the level of expression or the phosphorylation of the enzyme or the substrate availability, is significantly modified. In addition, the overall expression of $cPLA_2$, p11 and AxII (annexin II), the natural ligand of p11, is unchanged as a function of cell confluence. Co-immunoprecipitation experiments show that $cPLA_2$, p11 and AxII can interact. The stoichiometry of these complexes is strikingly dependent on cell confluence, since p11 was only faintly associated with $cPLA_2$ and AxII in non-confluent cells. Therefore our results suggest that the decreased $cPLA_2$ activation does not depend on the absolute level of p11 expression, but on its direct interaction with $cPLA_2$, which is increased after confluence.

EXPERIMENTAL

Cell lines and culture

Reagents for cell culture were from Life Technologies (Cergy-Pontoise, France). MDCK type II cells [28] were grown in Dulbecco's modified Eagle's medium, supplemented with 10 % (v/v) heat-inactivated (56 °C, 30 min) FCS (foetal calf serum), 100 units/ml penicillin and 0.1 mg/ml streptomycin (FCS-medium); Caco-2 cells (colon carcinoma-2 cells) [29,30] were grown in Dulbecco's modified Eagle's medium, supplemented with 20 % (v/v) heat-inactivated FCS, 1 % (v/v) non-essential amino acids, 100 units/ml penicillin and 0.1 mg/ml streptomycin.

MDCK type II cells were plated at 2000 cells/cm^2 and Caco-2 cells were plated at 15 000 cells/cm^2. They were used either 3 days after plating (non-confluent cells, i.e. 30–50 % confluence) or 11 days after plating (stationary cells). Confluence was reached 5 days after plating.

AA release

MDCK type II and Caco-2 cells, cultured in 6-well plates, were labelled with 0.5 μCi/ml [^{3}H]AA (Amersham Biosciences, Saclay, France) for 18 h at 37 °C. Cells were then washed three times with PBS containing 0.2 % NEFA [non-esterified fatty acid ('free')]-free BSA (Sigma, St Quentin-Fallavier, France). Cells were incubated in the presence of 50 μM BEL (bromoenol lactone suicide substrate; Calbiochem, Strasbourg, France), an $iPLA_2$ inhibitor. After 30 min, BEL was removed and replaced by serum-free medium supplemented with 5 μM calcium ionophore A23187 (Sigma) and 1 μM PMA (Calbiochem) or with 5 units/ml thrombin (Sigma) or 1 mM ATP (Sigma) or vehicle.

In some experiments, cells were incubated with 50 μM BEL, then with 200 μM $AACOCF_3$ (arachidonyltrifluoromethyl ketone; Calbiochem), a $cPLA_2$ inhibitor, for 30 min at 37 °C and finally with A23187 and PMA. Under these conditions, the inhibitors had no effect on cell viability as measured by Trypan Blue exclusion.

Aliquots of the medium were withdrawn at the time indicated, centrifuged at 10 000 ***g*** for 3 min at room temperature (20 °C) to remove cell debris, and the supernatants were counted for radioactivity by liquid scintillation. Cells, scraped in 500 μl of distilled water, were washed twice with PBS and 0.2 % NEFA-free BSA, and counted for radioactivity by liquid scintillation.

Release of total fatty acids

Unlabelled MDCK type II cells, cultured in 6-well plates, were stimulated with 5 μM A23187 and 1 μM PMA for 60 min. Total NEFAs were extracted from medium and from cells (scraped in 1 ml of distilled water after three washes with ice-cold PBS and 0.2 % NEFA-free BSA) following a modified procedure of Dole, methylated by diazomethane and quantified by GC-MS [20]. We present only NEFAs released into the medium because we found no detectable variation of NEFAs released within the cells.

Enrichment in AA

AA (Sigma) was dissolved in 100 % ethanol and added to the complete culture medium at a final concentration of 25 μM [31]. MDCK type II cells were grown for 11 days in complete medium supplemented with AA or vehicle, prepared and changed every day.

AA content

MDCK type II cells cultured in 25 cm^2 flasks were washed three times with ice-cold PBS and 0.2 % NEFA-free BSA and scraped in 1 ml of distilled water. An aliquot of lysate was withdrawn and the protein content was determined by the method of Lowry using BSA as standard. Cell phospholipids were extracted by the Bligh and Dyer method [32] followed by derivatization by methylation and separated by GC.

Immunoblot analysis

Non-confluent cells, cultured in 175 cm^2 flasks, or stationary cells, cultured in 75 cm^2 flasks, were washed three times with ice-cold PBS, scraped in ice-cold lysis buffer A (100 mM KCl/1 mM EGTA/1.1 mM $CaCl_2$/1 μg/ml leupeptin/1 μg/ml PMSF/20 mM Tris/HCl, pH 7.4) [33] and passed ten times through a 23-gauge needle. After sonication, the protein content of cell lysates was determined by the method of Lowry using BSA as standard.

Proteins were resolved by SDS/PAGE [7.5 % ($cPLA_2$), 10 % (AxII) or 15 % (p11) gels] under reducing conditions and transferred on to nitrocellulose membrane for immunoblot analysis. Blots were probed with 1 μg/ml mouse monoclonal anti-$cPLA_2$ antibody (SC-454; Santa Cruz Biotechnology, Le Perray en Yvelines, France) or 250 ng/ml mouse monoclonal anti-p11 antibody (AxII light chain; BD Transduction Laboratories, Le Pont de Claix, France) or 250 ng/ml mouse monoclonal anti-AxII antibody (BD Transduction Laboratories), followed by horseradish-peroxidase-conjugated anti-mouse IgG antibody (0.01 ng/ml; Jackson ImmunoResearch Laboratories, Montluçon, France) and detected with ECL® luminescence system (Amersham Biosciences).

Phosphorylation analysis

Non-confluent cells, cultured in 175 cm^2 flasks, or stationary cells, cultured in 75 cm^2 flasks, were washed three times with ice-cold PBS, scraped in ice-cold lysis buffer B (lysis buffer A containing anti-phosphatases: 0.2 mM Na_3VO_4, 10 mM β-glycerophosphate, 10 mM NaF and 5 mM dithiothreitol). After sonication, the protein content of cell lysates was determined by the method of Lowry using BSA as standard.

Proteins were resolved by SDS/PAGE [7.5 % ($cPLA_2$) gel] under reducing conditions and transferred on to nitrocellulose membrane for immunoblot analysis. Blots were probed with 1 μg/ml mouse monoclonal anti-$cPLA_2$ antibody (SC-454; Santa Cruz

Biotechnology) followed by horseradish-peroxidase-conjugated anti-mouse IgG antibody (0.01 ng/ml) (Jackson ImmunoResearch Laboratories) and detected with ECL® luminescence system (Amersham Biosciences).

Immunoprecipitation

Preparation of Protein A–Sepharose beads

Protein A–Sepharose beads (Amersham CL-4B) were washed once with ice-cold distilled water and twice with ice-cold PBS and finally resuspended in ice-cold PBS (final concentration, 1 mg/ml). For immunoprecipitation of mouse antibody (p11 and AxII), Protein A was preincubated with rabbit polyclonal anti-mouse antibody (4.5 μg/70 μg of Protein A–Sepharose beads; ICN, Orsay, France) 4 h at 4 °C. After centrifugation (at 11 000 ***g*** for 10 min at 4 °C), Protein A and Protein A coupled with rabbit anti-mouse antibody were washed twice with ice-cold PBS. Just before use, Protein A and Protein A coupled with rabbit anti-mouse antibody (70 μg of Protein A/sample) were washed twice and resuspended in lysis buffer.

Preparation of cell lysates

Non-confluent or stationary cell lysates were prepared as described previously. Lysates were pre-cleared by incubation with 40 μg of Protein A–Sepharose beads for 1 h at 4 °C. Lysates were centrifuged (at 11 000 ***g*** for 15 min at 4 °C) and the supernatants were subjected to immunoprecipitation.

Immunoprecipitation

Cleared supernatants were incubated with 1.5 μg of rabbit anti-cPLA$_2$ antibody (SC-438; Santa-Cruz Biotechnology) or 1 μg of mouse monoclonal anti-p11 antibody (BD Transduction Laboratories) or 1 μg of mouse monoclonal anti-AxII antibody (BD Transduction Laboratories) for 18 h at 4 °C, then with 70 μg of Protein A or 70 μg of Protein A coupled with rabbit anti-mouse antibody for 4 h at 4 °C. After two washes with ice-cold PBS and one wash with ice-cold Tris-buffered saline, beads were boiled with 10 μl of 10 % SDS for 5 min to elute proteins, and the beads were removed by centrifugation. The resulting supernatants were resolved by SDS/PAGE (7.5 % for cPLA$_2$, 10 % for AxII and 15 % for p11) and transferred to nitrocellulose membrane for immunoblot analysis. Blots were probed with mouse monoclonal anti-cPLA$_2$ or anti-p11 antibodies or 0.4 μg/ml goat polyclonal anti-AxII (SC-1924; Santa Cruz Biotechnology) followed by horseradish-peroxidase-conjugated anti-mouse or anti-goat IgG antibody (0.01 ng/ml; Jackson ImmunoResearch Laboratories) and detected with ECL® luminescence system (Amersham Biosciences).

Immunofluorescence

MDCK type II cells were plated on glass coverslips in 6-well plates and grown for 3 (non-confluent cells) or 11 days (stationary cells). Cells were washed three times with ice-cold PBS, and fixed with 4 % (w/v) paraformaldehyde for 10 min. Cells were washed three times with ice-cold PBS and incubated in PBS with NH_4Cl (50 mM) three times for 5 min. Cells were permeabilized with 0.075 % (w/v) saponin in PBS/NH_4Cl for 20 min.

Antibodies were used as indicated in Table 1. Rabbit pAb (polyclonal antibody) anti-cPLA$_2$ (SC-438) and mouse mAb (monoclonal antibody) anti-cPLA$_2$ (SC-454) were from Santa Cruz Biotechnology. Mouse mAb anti-p11 was from Transduction Laboratories, and rabbit pAb anti-AxII was a gift from Jesus Ayala-Sanmartin (INSERM U538, Paris, France). All fluorochrome-conjugated antibodies were from Jackson ImmunoResearch Laboratories.

Table 1 Antibodies and incubation times used for immunofluorescence experiments

Cells were incubated 18 h at 4 °C with Ab1, and for 1 h at room temperature for Ab2, Ab3 and Ab4. cPLA2 (green), and p11 and AxII (red).

	cPLA2–p11	cPLA2–AxII
Ab1	Rabbit pAb anti-cPLA2 (4 μg/ml)	Mouse mAb anti-cPLA2 (5 μg/ml)
Ab2	FITC-donkey pAb anti-rabbit (4 μg/ml)	FITC-donkey pAb anti-mouse (4 μg/ml)
Ab3	Mouse mAb anti-p11 (5 μg/ml)	Rabbit pAb anti-AxII 1/100
Ab4	Cy3-donkey pAb anti-mouse (4 μg/ml)	Cy3-donkey pAb anti-rabbit (4 μg/ml)

Cells were incubated with Ab1. After three washes with ice-cold PBS/NH_4Cl, cells were incubated with the corresponding fluorochrome-conjugated antibody Ab2, then with Ab3 and the corresponding fluorochrome-conjugated antibody Ab4. After the last antibody, cells were washed three times in ice-cold PBS/NH_4Cl and incubated for 10 min with antifading 1,4-diazadicyclo[2,2,2]octane (DABCO, 100 mg/ml; Sigma). The mounting medium was Fluoprep (BioMérieux SA, France). Results were analysed by confocal microscopy.

Statistical analysis was performed using paired Student's *t* test ($0.005 < *P < 0.05$; $**P < 0.005$).

RESULTS

Stimulation of AA release by agonists is decreased when epithelial cells reach confluence

MDCK type II cells were plated at 2000 cells/cm^2 and maintained in FCS-supplemented medium as described in the Experimental section. As expected, the cell number increased as a function of time (Figure 1). However, as shown by the growth curve, cells were still actively dividing when they reached confluence on day 5. The density of cells in the monolayer reached a plateau only 10–11 days after plating, which corresponded to a dynamic equilibrium between cell death and cell division [34]. AA release was monitored during progression of non-confluent cells to confluence and later to a highly polarized and organized monolayer (stationary cells). Cells taken at various times after plating were incubated with [^{3}H]AA for 18 h to label the bulk of exchangeable pools. Since PLA$_2$ activation depends on an increase in intracellular calcium and phosphorylation [35], MDCK type II cells were treated with calcium ionophore A23187 together with PMA to produce a maximal stimulation of AA release (Figure 2C) [36]. An interesting observation depicted in Figure 1 was that this treatment was able to promote a strong increase of AA release over the basal level only in non-confluent cells. Then, the level of stimulation decreased rapidly after the cells reached a confluence. This decrease occurred when cells were still actively dividing, suggesting that it was not primarily related to the rate of cell proliferation. In the mean time, the basal level of AA release was not affected by the stage of growth. The time course of the stimulation by agonists was studied further. It showed that in non-confluent cells, AA release increased linearly with time, and reached a plateau between 30 and 60 min (Figure 2A), whereas a weak stimulation was observed in stationary cells. In further experiments, stimulation was performed for 60 min routinely. As expected, the decrease in AA release resulted in a decreased production of eicosanoids. Indeed, the increase in prostaglandin E$_2$ accumulated in cell medium after

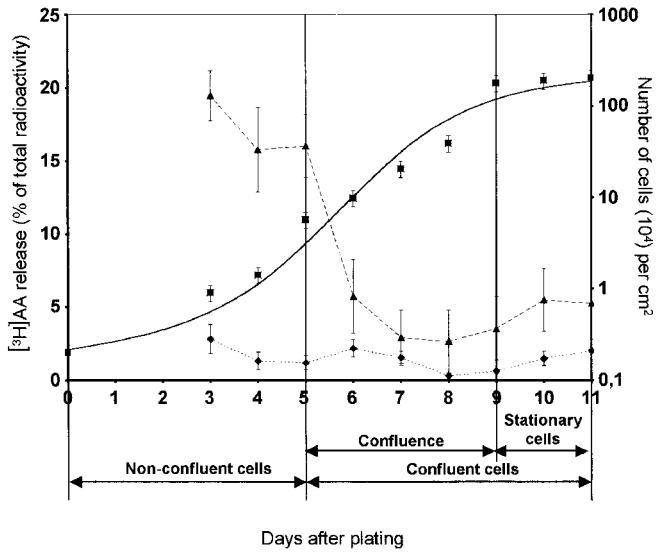

Figure 1 Decrease in AA in MDCK type II cells at confluence

MDCK type II cells were plated in 25 cm^2 flask. They were counted after trypsinization each day from day 3 until day 11 after plating. Results, expressed in number of cells/cm^2, are the means ± S.D. for one experiment performed in triplicate (logarithmic scale, solid line with square). MDCK type II cells (plated in 6-well plates) labelled with [^{3}H]AA for 18 h were stimulated with 5 μM A23187 and 1 μM PMA or vehicle for 60 min. At the end of incubation, aliquots of media were removed and cells were scraped in water. Aliquots of cells and media were counted for radioactivity. Results of AA release are expressed as a percentage of the total radioactivity incorporated. Values are the means ± S.D. for one representative experiment performed in triplicate (dotted line with lozenge, unstimulated cells; dotted line with triangle, stimulated cells).

stimulation with A23187 and PMA for 60 min was 8 times over basal level in non-confluent cells versus twice in stationary cells (results not shown).

To verify the confluence-dependent stimulation of PLA_2, another epithelial cell line, Caco-2 cells, derived from human colon carcinoma, was tested in non-confluent (day 3) and stationary phases (day 11). As shown in Figure 2(B), AA release from [^{3}H]AA-labelled Caco-2 cells was stimulated by A23187 and PMA only in non-confluent cells.

A23187 and PMA are pharmacogical agents, which elicit a rather strong and non-physiological response. To verify that the stimulation of AA release could be observed in more physiological conditions, we tested the effect of two agonists known to interact with membrane receptors in MDCK type II cells. Figure 2(C) shows that ATP and thrombin, acting through purinergic and thrombinergic G-protein-coupled receptors respectively and described previously in MDCK cells [37], significantly stimulate AA release from non-confluent cells, although to a much lower extent when compared with A23187 and PMA. In contrast, stationary cells exhibited a very low reactivity to ATP stimulation, whereas thrombin had no detectable effect. These results indicated that a physiological receptor-mediated AA release was efficient mainly in non-confluent cells and strongly decreased in stationary cells, as observed above with pharmacological agents.

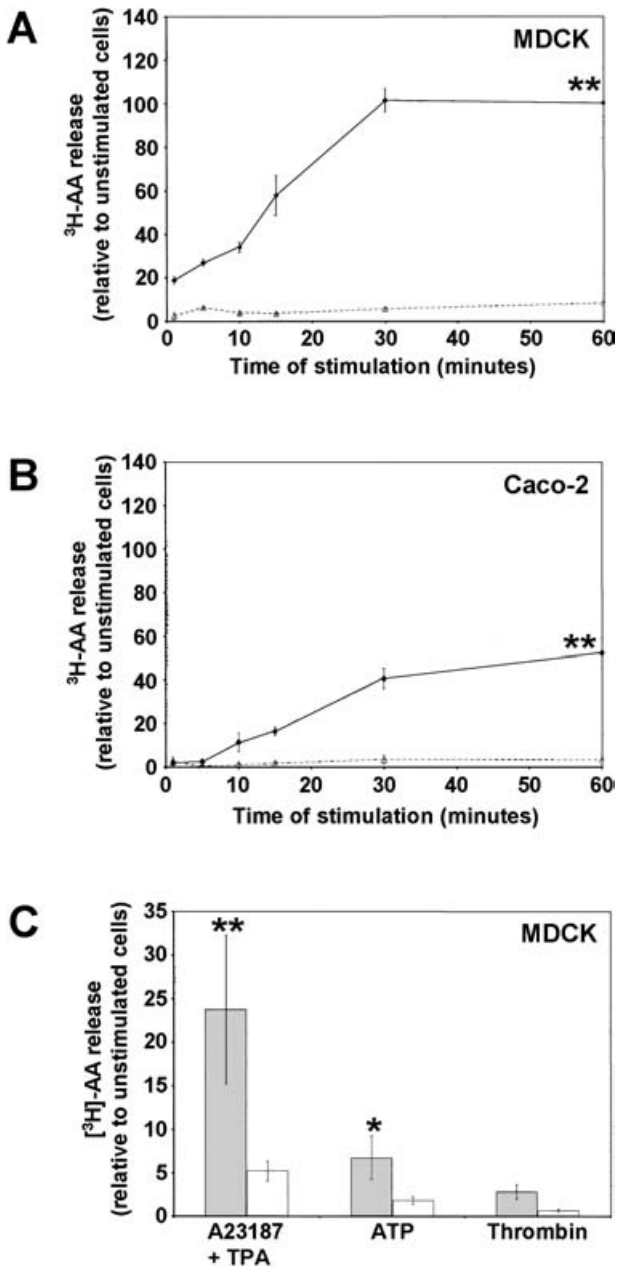

Figure 2 Decrease in AA release in another stationary epithelial cell line and in response to different agonists

MDCK type II and Caco-2 cells plated on 6-well plates were labelled with [^{3}H]AA. (**A**, **B**) Non-confluent (solid line) and stationary (dotted line) MDCK type II (**A**) and Caco-2 cells (**B**) were stimulated with 5 μM A23187 and 1 μM PMA or vehicle. An aliquot of the medium containing effectors was taken at 1, 5, 10, 15, 30 and 60 min of stimulation. At the end of incubation, the medium was removed and the cells were scraped in water. Aliquots of cells and media were counted for radioactivity. Results are expressed as AA released by stimulated cells relative to unstimulated cells. Values are means ± S.D. for one representative experiment performed in triplicate. (**C**) Non-confluent (grey bars) and stationary (white bars) MDCK type II cells were stimulated with 5 μM A23187 plus 1 μM PMA for 60 min, 1 mM ATP for 30 min or 5 units/ml thrombin for 30 min or vehicle. At the end of incubation, an aliquot of the medium was removed and the cells were scraped in water. Aliquots of cells and media were counted for radioactivity. Results are expressed as AA released by stimulated cells relative to unstimulated cells. Values are means ± S.E.M. for 3–9 independent experiments performed in triplicate.

AA is specifically released by $cPLA_2$ in MDCK type II cells stimulated with agonists

Since different PLA_2s could release AA, it was important to assess which one was responsible for the above observation. To this end, we performed two types of experiments. First, we studied the pattern of the fatty acids released in to the medium by GC–MS. As shown in Figure 3(A), the three main fatty acids released by MDCK type II cells under stimulation were oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$) and AA ($C_{20:4}$). Under these conditions, no

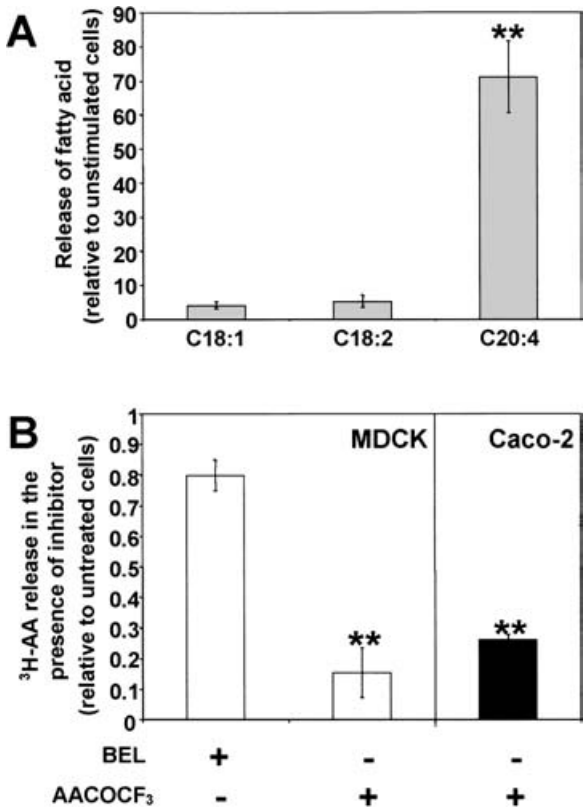

Figure 3 Specific AA release by cPLA$_2$ in MDCK type II cells

(**A**) Non-confluent MDCK type II cells were stimulated with 5 μM A23187 plus 1 μM PMA for 60 min. The three main fatty acids released in medium were quantified by GC–MS ($C_{18:1}$, oleic acid; $C_{18:2}$, linoleic acid; $C_{20:4}$, AA). Results are expressed as fatty acid released by stimulated cells relative to unstimulated cells. Values are means $\pm$ S.E.M. for three independent experiments performed in triplicate. (**B**) Effects of PLA_2 inhibitors on AA release. Non-confluent cells were labelled with [^{3}H]AA. They were treated with 200 μM $AACOCF_3$ (MDCK and Caco-2 cells) or 50 μM BEL (MDCK) for 30 min, then stimulated with 5 μM A23187 and 1 μM PMA for 60 min. At the end of incubation, an aliquot of medium was removed and the cells were scraped in water. Aliquots of cells and medium were counted for radioactivity. Results were expressed as [^{3}H]AA released by stimulated cells in the presence of inhibitor relative to untreated cells. Values are means $\pm$ S.E.M. for three independent experiments performed in triplicate (white bars, MDCK type II; black bar, Caco-2).

significant variation of the intracellular concentration of NEFAs was observed. Strikingly, AA release was specifically increased (70 times in non-confluent cells and 20 times in stationary cells), whereas the release of any other fatty acid was essentially unaffected. Secondly, inhibitors were then used to assess further the specificity of the PLA_2 responsible for AA release. Figure 3(B) shows that BEL, a specific inhibitor for iPLA$_2$, has only a very slight inhibitory effect on AA release. In contrast, $AACOCF_3$, a selective inhibitor for cPLA$_2$, inhibits AA release by two-thirds in non-confluent (Figure 3B) and stationary (results not shown) MDCK type II cells. Similar results were obtained in non-confluent Caco-2 cells (Figure 3B). Given the kinetics of AA release, it was unlikely that sPLA$_2$ could be involved, and therefore this enzyme was not studied further. Together, these experiments showed that, in MDCK type II and Caco-2 cells, AA is mainly released by cPLA$_2$ under stimulation.

A decrease in AA content does not explain the down-regulation of AA release

A possible explanation for a decreased AA release in stationary cells could be that cPLA$_2$ substrate was decreased. It has been

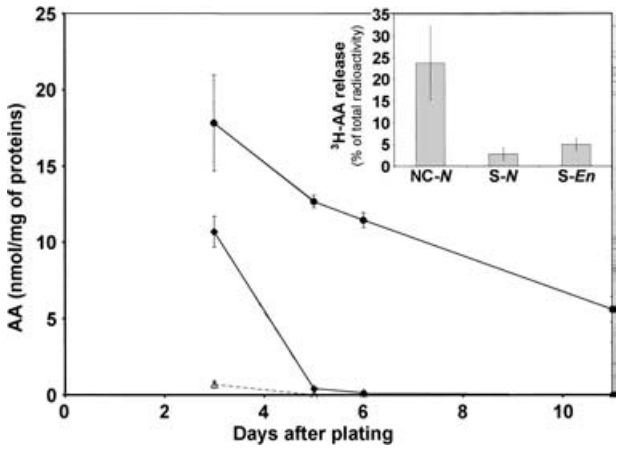

Figure 4 AA release and AA content in stationary cells

MDCK type II cells plated in 25 cm^2 flasks were cultured for 3–11 days. Total fatty acids were extracted on days 3, 5, 6 and 11 after plating. MDCK type II cells plated on 6-well plates were stimulated by 5 μM A23187 and 1 μM PMA or vehicle for 60 min on days 3, 5, 6 and 11 after plating. AA release (basal release, dotted line with triangle; stimulated release, solid line with lozenge) and AA content (solid line with circle) were determined by GC–MS. AA release and AA content (expressed in nmol/mg of proteins) were reported with respect to the protein content. Values are means $\pm$ S.D. for one representative experiment performed in triplicate. (Inset) [^{3}H]AA release in stationary cells enriched in AA. MDCK type II cells cultured for 11 days in FCS supplemented with 25 μM AA were then labelled with [^{3}H]AA for 18 h, stimulated with 5 μM A23187 and 1 μM PMA or vehicle for 60 min, and radioactivity in the medium and cells was counted. Results of AA release are expressed as a percentage of the total radioactivity incorporated (NC-N, non-confluent normal cells; S-N, stationary normal cells; S-En, stationary enriched cells). Values are means $\pm$ S.E.M. for three independent experiments performed in triplicate.

shown previously that cPLA$_2$, in contrast with other PLA$_2$s, was able to hydrolyse any arachidonyl phospholipid without selectivity towards its polar head [38]. Therefore AA content of whole-cell phospholipids was compared in non-confluent and stationary MDCK type II cells to evaluate whether a decreased level of cPLA$_2$ substrate may be responsible for the loss of its activation. When maintained in culture, stationary MDCK type II cells decreased their content of AA by an additional 40 %. This decrease occurred linearly with time in culture. However, as shown in Figure 4, the strong decrease in cPLA$_2$ stimulation observed when MDCK type II cells reached day 5 cannot be attributed to the decrease in AA content, since at that time, it was only decreased by 20 % when compared with day 3. To demonstrate further the absence of a relationship between AA content and cPLA$_2$ activation, MDCK type II cells were cultured in a medium supplemented with 25 μM AA for 11 days before labelling with [^{3}H]AA and stimulation. At the end of this treatment, stationary cells had reached a much higher level of AA (approx. 23 % of total fatty acids versus 7 % in non-enriched non-confluent cells; result not shown). Strikingly, this enrichment in AA only doubled [^{3}H]AA release under stimulation, which remained 5 times lower than in non-confluent cells (Figure 4, inset). This result clearly confirmed that cPLA$_2$ down-regulation in stationary MDCK type II cells was not due to AA deficiency.

cPLA$_2$ expression or phosphorylation is unchanged in non-confluent cells

Another explanation for the down-regulation of cPLA$_2$ could be a decrease in its expression or phosphorylation. Immunoblot analysis of non-confluent and stationary MDCK type II cells

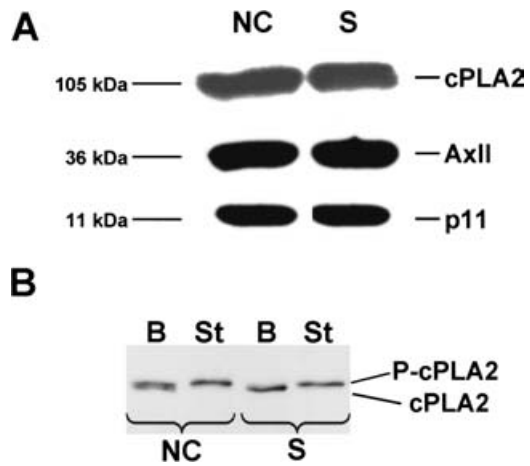

Figure 5 Expression of $cPLA_2$, p11 and AxII in non-confluent and stationary cells

(**A**) 50 μg (AxII or p11) or 200 μg ($cPLA_2$) of total cell lysates from non-confluent (NC) and stationary (S) MDCK type II cells were separated by SDS/PAGE and analysed by immunoblotting. The results are representative for 3 to 6 independent experiments. (**B**) Non-confluent (NC) and stationary (S) MDCK type II cells were stimulated by 5 μM A23187 and 1 μM PMA (St, stimulated cells), or vehicle (B, unstimulated cells) for 60 min. The cells were then washed and scraped in lysis buffer. Total cell lysates (200 μg) were resolved by SDS/PAGE and analysed by immunoblotting using monoclonal anti-$cPLA_2$ antibody that recognizes $cPLA_2$ and the phosphorylated form of $cPLA_2$ (P-$cPLA_2$). Results are representative for three independent experiments.

revealed no difference in the expression level of $cPLA_2$ (Figure 5A). To evaluate the phosphorylation of $cPLA_2$, we performed a more resolutive separation of proteins by increasing the migration time of SDS/PAGE. Under these conditions, the phosphorylated form of $cPLA_2$ appeared as a distinct band with a decrease in mobility. As shown in Figure 5(B), the phosphorylated band of $cPLA_2$ increased after stimulation in non-confluent cells as well as in stationary cells.

Interaction of $cPLA_2$ with p11 is increased in non-confluent cells

p11 is the only protein known to interact directly with $cPLA_2$ and to regulate its activity negatively [25,39]. Figure 5(A) shows that the expression level of p11 in whole cell lysate was similar in stationary and in non-confluent cells. Similarly, AxII, which interacts with p11, but has no effect on $cPLA_2$ activity [23], was unchanged.

To study the interaction between p11 and $cPLA_2$, we performed co-immunoprecipitation experiments with anti-$cPLA_2$, anti-p11 or anti-AxII antibodies, and immunoblots of the precipitated complex were developed. Figure 6 shows that p11 and AxII co-precipitated with $cPLA_2$. Interestingly, the amount of p11 immunoprecipitated with $cPLA_2$ was higher in stationary cells [lane 2 (stationary) compared with lane 1 (non-confluent)]. Conversely, immunoprecipitation of p11 revealed that more $cPLA_2$ co-precipitated in stationary cells [lane 4 (stationary) compared with lane 3 (non-confluent)]. In contrast, the amount of AxII immunoprecipitated with anti-$cPLA_2$ or anti-p11 antibodies was not significantly lower in non-confluent and stationary cells (lanes 1–4). Consistently, immunoprecipitation of AxII revealed the same amount of $cPLA_2$ and p11 in non-confluent and stationary cells [lane 6 (stationary) compared with lane 5 (non confluent)].

The main conclusion to be drawn from these results is that, although expressed at the same level in whole cells, $cPLA_2$

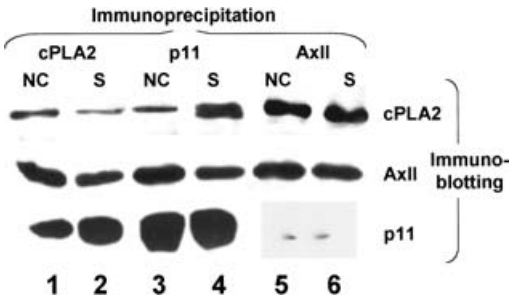

Figure 6 Co-immunoprecipitation of $cPLA_2$, p11 and AxII in non-confluent and stationary cells

Lysates from non-confluent (NC) and stationary (S) MDCK type II cells were incubated with rabbit polyclonal anti-$cPLA_2$, monoclonal anti-p11 or monoclonal anti-AxII antibodies and then with Protein A–Sepharose beads. After three washes, the beads were collected and the precipitated proteins were subjected to SDS/PAGE followed by immunoblotting with the corresponding antibodies (monoclonal anti-$cPLA_2$, monoclonal anti-p11 and polyclonal anti-AxII antibodies). Results are representative for three independent experiments.

displayed a different accessibility to p11, dependent on cell confluence.

This is confirmed by immunofluorescence studies (Figure 7). It appears that AxII and p11 are concentrated at the periphery near the plasma membrane in non-confluent cells (Figures 7a and 7g). In these cells, $cPLA_2$ is mainly located in the cytosol (Figures 7b and 7h). In stationary cells, the labelling near plasma membrane disappears for AxII and p11 (Figures 7d and 7j), and co-localization with $cPLA_2$ is strongly increased for p11 (Figure 7c compared with 7f).

DISCUSSION

Our results show that stimulation of AA release decreases when MDCK type II cells reach confluence. These results have been obtained in metabolically labelled cells and confirmed by GC–MS measurement of fatty acids released under stimulation. This decrease is not restricted to renal epithelial cells, as it is reproduced in an intestinal epithelial cell line, Caco-2. Interestingly, the decrease in AA release in stationary epithelial cells was observed both when cells were triggered with pharmacological agents and with receptor-mediated stimuli such as ATP or thrombin. This means that this decrease is not related to the amplitude of calcium changes nor to the number or type of membrane receptors. In this situation, AA release appeared as the main rate-limiting step in eicosanoid production since prostaglandin E_2 production was decreased in parallel with AA production in stationary cells when compared with non-confluent cells.

The release of free AA is triggered by hydrolysis of membrane phospholipids by PLA_2s. Among the wide variety of unrelated proteins constituting the PLA_2 family, $cPLA_2$ has been evidenced as the main enzyme involved in short-term stimulation of AA release [16,17]. In accordance with results reported previously for MDCK [37], the present analysis of the profile of fatty acids released and the use of specific inhibitors confirmed that $cPLA_2$ was actually responsible for the stimulus-induced AA release in MDCK type II cells.

A major factor limiting PLA_2 activity is the availability of its membrane substrate, namely AA-containing phospholipids. To verify if the down-regulation of AA release could result from a variation of AA availability at confluence, the level of AA was compared in MDCK type II cell membranes from non-confluent and stationary cells. It appeared that its relative content was decreased by 2.5 times at confluence. However, this decrease

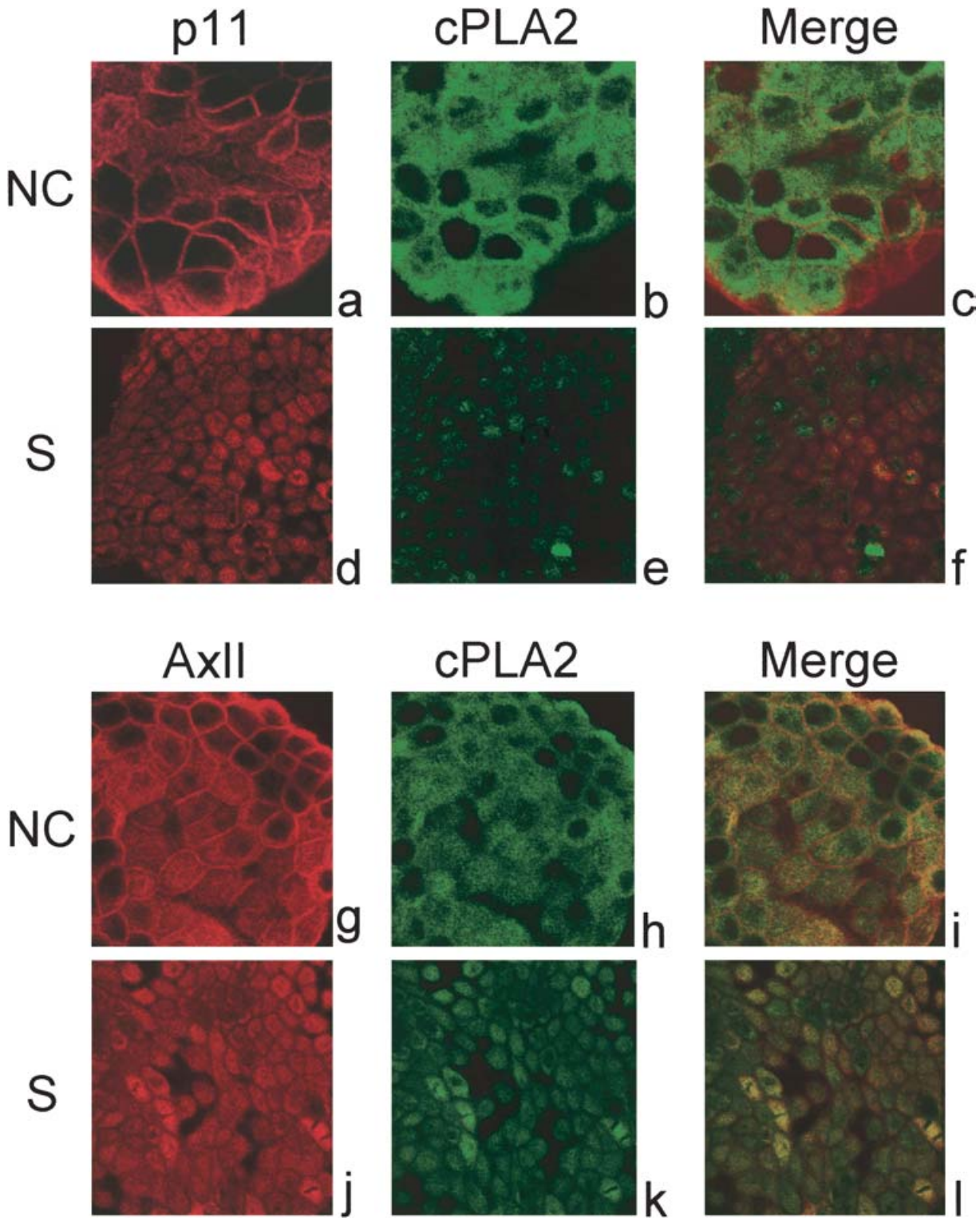

Figure 7 Increase in co-localization of $cPLA_2$ with p11 and AxII in stationary cells

Non-confluent (NC) or stationary (S) cells were treated as described in the Experimental section. They were labelled with anti-cPLA2 antibody (green) (b and h, non-confluent; e and k, stationary). Cells were then labelled with anti-p11 antibody (red) (a, non-confluent; d, stationary) or anti-AxII antibody (red) (g, non-confluent; j, stationary). Merges were presented in the last lane (c and f, non-confluent; i and l, stationary). Co-localization appears in yellow.

was not sufficient to explain the 10-fold decrease in stimulation of AA release in stationary cells. Control experiments were performed in stationary cells enriched in AA to verify if the level of AA release depended on the level of AA in whole cell phospholipids. As expected from previous studies, AA release was increased in AA-enriched stationary cells [31]. However, this increased release remains very low when compared with the release of AA observed in non-confluent cells. Therefore it seems

that the decreased AA release observed in stationary MDCK type II cells cannot be explained by a decreased AA availability.

Another possibility to explain the down-regulation of $cPLA_2$ activation is a change in proteins interacting with $cPLA_2$. $cPLA_2$ activation is prevented by its interaction with p11, a cytosolic protein which binds to the C-terminal domain of $cPLA_2$ to form an inhibitory complex [25,39]. Although the actual mechanism of $cPLA_2$ inhibition by p11 is unknown, the inhibition seems to follow the expression level of p11, which is limited for the formation of the inhibitory complex. Indeed, several agonists have been reported to regulate $cPLA_2$ activation through an up-regulation of p11 expression level in several epithelial cell types [25,39,40]. This is not the case in our cell model, where expression of both p11 and $cPLA_2$ remains constant when $cPLA_2$ activation is decreased. This would suggest that p11 availability for interaction with $cPLA_2$ should be controlled by interaction of p11 with other partners. Indeed, p11 is known to be tightly associated with AxII to form an heterotetramer $(AxII)_2(p11)_2$ [41]. The family of annexins binds phospholipids and membranes in a calcium-dependent manner [42]. However, the $(AxII)_2(p11)_2$ heterotetramer is capable of inhibiting $cPLA_2$ activity *in vitro*, which is not the case for AxII alone, in contrast with other members of annexin family [23]. Our results are the first to suggest that in living epithelial cells AxII, p11 and $cPLA_2$ might be associated. This association is increased in stationary cells and efficiently inhibits $cPLA_2$ activation. Since the level of AxII co-immunoprecipitated with $cPLA_2$ also remains constant in non-confluent and stationary cells, this means that AxII is not the main factor for a decreased $cPLA_2$ activation. Rather, this clearly indicates that the critical regulating event for $cPLA_2$ activation depends on its interaction with p11, which is strongly increased in stationary cells.

What can explain a change in the stoichiometry of $cPLA_2$/p11 interaction with a constant expression of all the three partners as a function of cell confluence? It has been established that the $(AxII)_2(p11)_2$ heterotetramer is mainly found in the cytosol, beneath the plasma membrane under resting conditions [42], in accordance with what we observed in non-confluent cells (Figures 7a and 7g). Our results show that, after confluence, AxII and p11 leave the cell periphery to relocalize in the cytosol. This event is consistent with the observed increased affinity for $cPLA_2$.

Two changes in $cPLA_2$ regulation have been previously suggested to explain the decrease in AA release observed in other cell types as a function of cell proliferation: a decrease in $cPLA_2$ expression in smooth-muscle cells [4] and a decrease in $cPLA_2$ phosphorylation in endothelial cells [6]. In contrast with these studies, we found no difference in the level of expression or in the apparent level of phosphorylation of $cPLA_2$ between stationary and non-confluent cells. The main differences between our work and these studies depend on the fact that we analysed epithelial cells and that we pay attention to the formation of cell–cell contacts (i.e. confluence) rather than to cell proliferation. Indeed confluent MDCK type II cells continue to grow after confluence to establish a dense monolayer characteristic of differentiated epithelia. It appears that $cPLA_2$ is negatively regulated in MDCK type II cells when these cells establish cell–cell contacts and not through a decrease in cell proliferation, which is observed much later after confluence and seems to have no further effect on $cPLA_2$ activation.

It is well known that the plasma membrane of cultured epithelial cells reaching confluence undergoes various structural and functional modifications, including the formation of adherent and tight junctions, which are one of the main features of an epithelial monolayer. These changes result in a profound remodelling of membrane lipid composition, including the formation of various microdomains with different local concentration of cholesterol, sphingolipids and phospholipids. Our current hypothesis is that when the formation of these microdomains in epithelial cells reaches confluence, an imbalance in the affinity of AxII/p11 for $cPLA_2$ against membrane takes place, which will explain the decrease in $cPLA_2$ activation.

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